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## Research article

# Antibiotic resistance genes in farm animal slaughterhouse wastes in Al-Dewanyiah province, Iraq

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## Abstract

Environment represents as a reservoir for emerging and disseminating of antibiotic resistance genes (ARGs). Slaughterhouse waste water is one of the important sources of these genes even after treatment processes. This study was conducted to evaluate role of farm animal slaughterhouse in distribution of antibiotics resistance in Al-Dewanyiah, Iraq. A total of eighty samples were collected from the central farm animal slaughterhouse. The detection was based on four mobile genetic elements and nine antibiotic resistance genes. Presences of mobile genetic elements (MGEs) were detected in the tested samples. Transposon (Tn3) were found in 80% of the tested samples while integrons class 1, 2 and 3 were in 56%, 24% and 1.6% respectively. The results also showed high frequency of tetracycline resistance genes in the tested samples with great relative abundance 60%. Genes encoding the resistance to quinolone, aminoglycoside and β-lactamases (mecA, CITM & OXY-1-1) were found in 31.2%, 10%, 30%, 12%, and 10% respectively. Furthermore, concentrations of ARGs and MGEs per ml of sample were tested using qPCR. Genes encoding for tetracycline resistance and transposon (Tn3) were found in higher concentration $P<0.05$ (copy number) per ml of slaughterhouse wastewater comparing with selected genes. Quantification of ARGs and MGEs in the slaughterhouse wastes indicated that those wastes represent as a hotspot for dissemination of antibiotic resistance to the environment. Low darning and no treatment for slaughterhouse wastes might increase the abundance of ARGs and resistant bacteria in the natural environment.

**Keywords**: Antibiotic resistance, Antibiotic resistant genes, Public health, Waste water & Slaughter houses.

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# INTRODUCTION

The overuse of antibiotics in the treatment of human and animal diseases lead to emerge and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment (Rather et al., 2017). ARGs usually associated with mobile genetic elements (MGEs) in the bacterial cell. MGEs include transposon; plasmid and Integron can transfer between bacterial cells via horizontal gene transformation (HGT). This lead to transmit of antibiotic resistance from resistant to sensitive bacteria (Martinez, 2009; Oliveira et al., 2017). Antibiotic resistant bacteria can cause more clinical problems after the infection than the sensitive bacteria as in case of methicillin-resistant Staphylococcus aureus (MRSA) infection, about 64% deadly more than non-resistant strains (WHO, 2019).

ARGs and antibiotic residues may transfer through the environment which is an important source behind antibiotic resistance problem in human and animals (Waseem et al., 2017). Environment elements consider as a reservoir to the ARGs and resistant bacteria (Martinez, 2009; Wright, 2010; Pepper et al., 2018). Wastewater of the farm animal slaughterhouse is one of the important sources of ARGs. Releasing of this type of wastewater to river without treatment can cause wide spread of ARGs and ARB in animal and human environments (Kim and Aga, 2007; Martinez, 2009; Cantas et al., 2013).

Further studies are needed to understand the pattern of ARGs emerging and spreading in the environment and factors that effect on horizontal transmission of ARGs. Currently, molecular methods are important tools can be used to detect wastewater contamination with antibiotic resistance genes such as PCR and real-time PCR (Heuer and Smalla, 2007; Walsh et al., 2011). These techniques were used in this study to identify availability and spreading of ARGs in slaughterhouse wastewater and to recognise the most common types of ARGs can be related with the animal wastes and intestinal contents. Furthermore, the current study can put a spot-on the necessity of restrict use of antibiotics in clinic and agriculture. Also, referring to the importance of treating the wastewater from slaughter houses to minimise risk of emergence and spread of antibiotic resistance.

# MATERIALS AND METHODS

## Samples collection

Wastewater samples were collected from wastewater drainage canal of farm animal (cattle& sheep) central slaughterhouse in Al-Dewanyiah town, south of Iraq. Eighty Samples were collected during the winter of 2022. 25 ml of wastewater were collected using 50 ml sterile falcon tube. Wastewater samples were filtered using 0.2µm filter membrane. The flow through was discarded and filter membrane was stored at 4°C until DNA extraction.

## DNA extraction

Metagnomic DNA was extracted from wastewater samples using Soil DNA Isolation plus Kit (Norgen®, Canada) according to the manufacturer's instructions with some modification. Quality and concentration of the extracted DNA were primarily estimated using Nano drop and agarose gel electrophoresis method. The extracted samples were stored at -20°C for further tests.

### Primers, PCR and qPCR

Abundance of mobile genetic elements (MGEs) and antibiotic resistance genes (ARGs) in the wastewater samples that collected from the farm animal slaughterhouse were tested using 13 primers. The primers belong to nine ARGs (tetO; tetK; erm(B); msr(A); aadA, qnrB; mecA ; CITM & OXY-1-1) and four types of MGEs (Tn3; ;Intl1; Intl2& Intl3)) as described in the results section and (Table 1). The primers were synthesized by Macrogen (Korea) and designed according to previous study (Waseem et al., 2019). Presence of ARGs and MGEs in the samples was detected using conventional PCR. High fidelity DNA polymerase (Phusion®, NEB England) and specific primers (Table 1) were used to amplify the target genes in the tested samples.

Quantities of the target genes in the samples were estimated using qPCR. The Applied Biosystems™ StepOne™ Real-Time PCR System was used to perform the DNA amplification in 50µL of the reaction. The reaction generally consists from DNA sample, SYBER green and primers of the targets. qPCR assay was carried out in triplicates.

## Data analysis

Graphs and statistical analysis were performed using GraphPad Prism 5 software. Experimental data were analysed using one-way ANOVA. $P< 0.05$ was considered as a significant. Microsoft excel was used to calculate and analyse the qPCR data.

**Table 1** The primers used to amplification and quantification of ARGs MGEs

| Primer name | Sequence | Ref. |
|---|---|---|
| Tet(O)F | AACTTAGGCATTCTGGCTCAC | Jeric et al., 2002 |
| Tet(O)R | TCCCACTGTTCCATATCGTCA | |
| Tet(K)F | TATTTTGGCTTTGTATTCTTTCAT | Zhao et al., 2018 |
| Tet(K)R | GCTATACCTGTTCCCTCTGATAA | |
| erm(B)F | GAACACTAGGGTTGTTCTTGCA | Neher et al., 2016 |
| erm(B)R | CTGGAACATCTGTGGTATGGC | |
| msr(A)F | TGCTGACACAATTTGGGAT | Lee et al., 2011 |
| msr(A)R | GAGCAGCCTTCTCAACC | |
| aadA1F | TTTGCTGGTTACGGTGAC | Waseem et al., 2017 |
| aadA1R | GCTCCATTGCCCAGTCG | |
| qnrBF | GATCGTGAAAGCCAGAAAGG | Waseem et al., 2017 |
| qnrBR | ACGATGCCTGGTAGTTGTCC | |
| mecAF | AAAAAGATGGCAAAGATATTCAA | Waseem et al., 2017 |
| MecAR | TTCTTCGTTACTCATGCCATACA | |
| CITMF | TGGCCAGAACTGACAGGCAAA | Aryal et al., 2020 |
| CITMR | TTTCTCCTGAACGTGGCTGGC | |
| oxy1F | CGTTCAGGCGGCAGGTT | Waseem et al., 2017 |
| oxy1R | GCCGCGATATAAGATTTGAGAATT | |
| Tn3F | GCTGAGGTGTTCAGCTACATCC | Nicolas et al., 2015 |
| Tn3R | GCTGAGGTAGTCACAGGCATTC | |
| Int1-F | GCCTTGATGTTACCCGAGAG | Toubiana et al., 2021 |
| Int1-R | GATCGGTCGAATGCGTGT | |
| Int2-F | TGCTTTTCCCACCCTTACC | Waseem et al., 2017 |
| Int2-R | GACGGCTACCCTCTGTTATCTC | |
| Int3-F | GCCACCACTTGTTTGAGGA | Toubiana et al., 2021 |
| Int3-R | GGATGTCTGTGCCTGCTTG | |

## RESULTS

Eighty wastewater samples were collected during the winter months from the central slaughterhouse of farm animal in the Al-Dewanyiah city in 2022. Sterile swaps were used to collect the samples. According to previous study (Mustafa et al., 2017) the samples were treated with aluminium chloride (AlCl3) in order to remove the humic acid which may inhibit or interfere with the DNA polymerase enzyme, then DNAs were extracted according to manufacturer instructions.

The Presence of ARGS and MGEs in the collected samples was detected using conventional PCR. Nine types of antibiotic genes were tested, including two genes encoding to the tetracycline resistance (tetO& tet(K)), two genes for erythromycin resistance (erm(B)& msr(A)), aminoglycoside resistance gene (aadA), quinolone resistance gene(qnrB) and three types of β-lactamases genes (mecA, CITM & OXY-1-1).

Tetracycline, aminoglycosides and erythromycin inhibit bacterial growth by interfering with protein synthesis (Tetracycline and aminoglycosides inhibit the 30S ribosomal subunit while erythromycin inhibit the 50S rns), two genes that encoding to tetracycline resistance were tested (tetO& tet (K)). TetO gene encoding to ribosomal protection tetracycline resistance protein, the gene confers resistance to tetracycline by releasing of the antibiotic from the ribosome. tet (K) gene encoding to transporter protein (efflux pump) which responsible for effluxing of tetracycline out of bacterial cell. The erm (B) & msr (A) encoding to erythromycin resistance. erm (B) gene encoding to 23S rRNA methyltransferase enzyme which confer the resistance to erythromycin by target modification. msr(A) gene encoding transporter protein belong to ABC transporter protein family. aadA gene encoding to aminoglycoside resistance by inactivation of antibiotics (Kapoor et al., 2017).

Quinolones a class of antibiotics that inhibit bacterial growth by inhibition of nucleic acid synthesis. qnrB encoding to quinolones resistance by preventing these type of antibiotics to bind to their target. mecA, CITM & OXY-1-1 encoding resistance to β-lactam antibiotic by inhibiting of penicillin binding protein which involves in synthesis of cell wall peptidoglycan (Kapoor et al., 2017; Yao et al., 2019).

As had shown in Figure 1, tetracycline recorded high frequent availability in the tested samples where one or both tetracycline genes recorded in 48 out of 80 samples (60%). PCR results also showed that quinolone and methicillin resistance genes were existed in about 39 samples (31.2%).

However, aminoglycoside and β-lactamases genes showed less frequent presence in the examined samples while no samples were positive to one or both of erythromycin genes. In addition, the results showed that antibiotic resistance genes are mostly associated and carried by mobile genetic elements and these elements have a critical role in dissemination of ARGs to the natural environments.

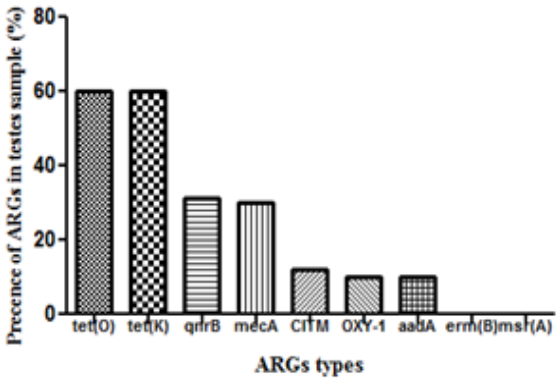



**Figure 1** The presence of ARGs in slaughterhouses wastes. Nine primers were used to amplify the selected resistance genes using PCR. Were tetO& tetK are tetracycline resistance gene. erm(B)& msr(A) are erythromycin resistance gene. aadA is aminoglycoside resistance gene (qnrB) is quinolone resistance gene and mecA, CITM & OXY-1-1 are β-lactam resistance gene.

Presence of four types of MGEs in the slaughterhouse wastewater samples were tested by PCR. The MGEs including transposon (Tn3) and Integrons (Intl1, Intl2& Intl3) were also detected. As shown in Figure 2, 64 out of 80 (80%) samples were positive to Tn3. While 56, 24 and 1.6% of Intl1, Intl2 & Intl3 respectively were found in the tested samples. Overall, MGEs were found in 76% of the slaughterhouse wastewater samples (Figure2).

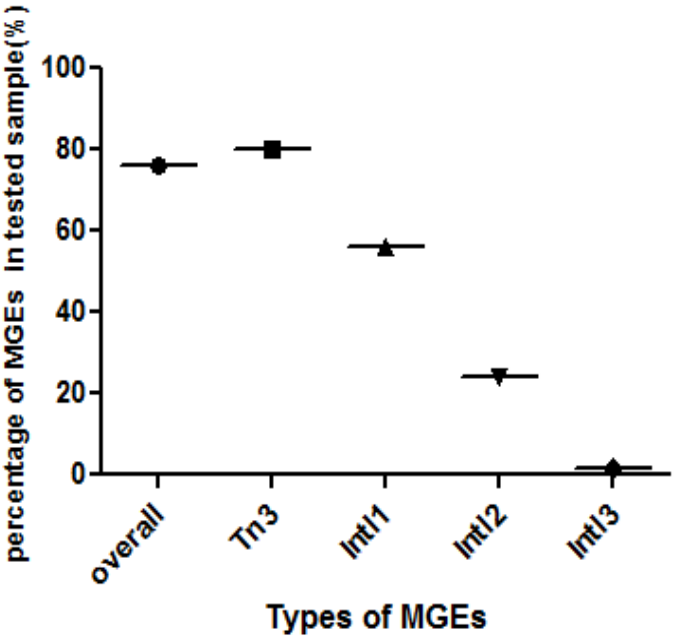



**Figure 2** presence of MGEs in slaughterhouses wastes. Four primers were used to amplify the selected mobile genetic elements using PCR. Overall refer to the total MGEs. Tn3 is transposon. Int1, Int1and Int1 is Integron class one, two and three respectively.

Concentrations of ARGs and MGEs in the tested samples were estimated using qPCR. As shown in Figure 3, tetracycline genes recorded high copies (P< 0.5) per ml of wastewater sample as a compare with the other tested resistance genes followed by aminoglycoside and quinolone, while β-lactamases genes showed low concentrations in the tested samples. Figure 4 showed that the integrons class 1 recorded high concentration (P< 0.5) as a compare to the other mobile genetic elements followed by transposon Tn3, while Integrons class 3 showed low concentration in the wastewater samples.

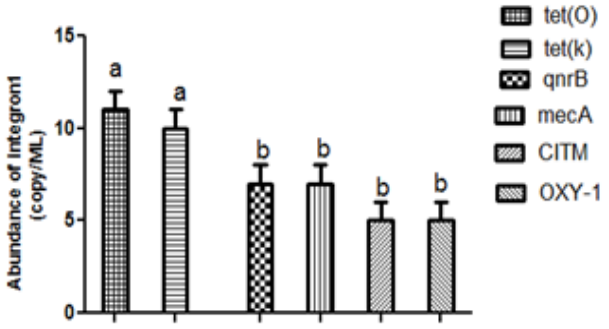



**Figure 3** Concentrations of ARGs in slaughterhouses wastes. The data represent copy number of the selected genes per ml of the sample. qPCR was used to measure concentrations of the selected genes. Letter (a, b) refer to significant differences at P<0.05.

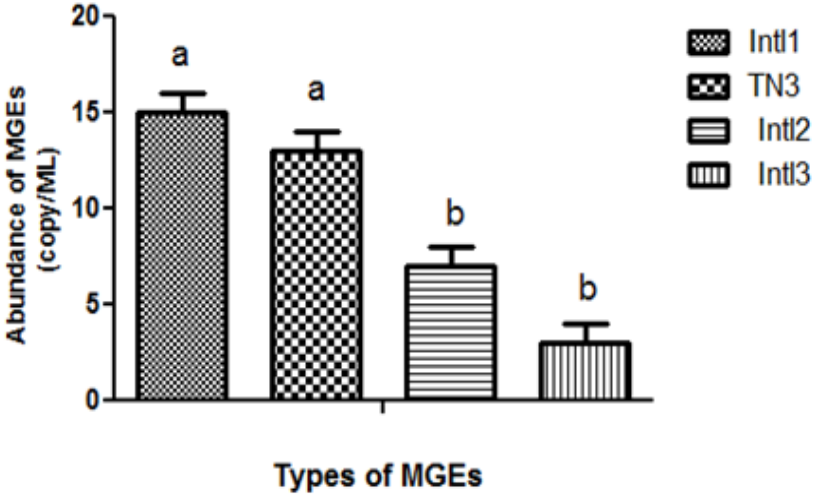



**Figure 4** Concentration of MGEs in slaughterhouses wastes. The data represent copy number of the selected genes per ml of sample. qPCR were used to measure concentration of the selected genes. Letter (a, b) refer to significant differences at P<0.05.

# DISCUSSION

It is well documented that environmental elements play a crucial role in dissemination of antimicrobial resistance. A livestock slaughterhouse waste is one of the sources of biological contaminants, including antibiotic resistance genes. From this source ARGs emerge and transfer to the animal and human environment (Kunhikannan et al., 2020; Saekhow et al., 2021). Mobile genetic elements are usually associated with the antibiotic resistance gene and antibiotic resistant bacteria . Silva and her colleague (2022) found that MGEs are associated with 77% of the tested ARGs in Pasteurellaceae Species. Several studies documented that MGEs promote spreading of ARGs and ARB in the natural environment. MGEs involve in the spreading of ARGs among different species of bacteria via HGT also it can be used as biomarker for assessment dissemination of antibiotic resistance in the environments. Untreated wastewater or sewage that has a high concentration of MGEs represents as a hotspot for dissemination of ARGs and it is considered as a potential risk factor for human and animal health (Che et al., 2019; Zhao et al., 2023).

In this study, presence of ARGs & MGEs in the farm animal slaughterhouse was investigated. Also, our study primarily assessed role of the farm animal slaughterhouse in dissemination of ARGs to the environment. Recently, ARGs are considered as emerging contaminants and many studies documented that ARGs can be found in soil, ground and surface of water and in the air. Also, ARGs usually associated with MGEs such as transposons, Integrons and plasmids. These elements promote transmission of those genes among bacteria (He et al., 2020). In addition, ARGs can enter a new host cell (bacteria) by horizontal gene transmission resulting in resistant bacteria. These bacteria can widely distribute in the environment with the aid of antibacterial residues which allow resistant bacteria to grow and prevent the others (selective pressure) (Colavecchio et al., 2017). Moreover, resistant bacteria are responsible for increasing of mortality in human and animals after bacterial infection as a result of treatment failure (Esperón et al., 2018).

Interestingly, many studies have suggested the relationship between heavy using of antibiotics and the presence of ARGs in the environment elements (soil and water) (Esperón et al., 2018). These studies partially agree with our study where we found that genes encoding to tetracycline were frequently detected in tested samples as a compare with other ARG. Tetracycline is a broad-spectrum antibiotic and widely used in veterinary medicine in the area where the study was performed. However, no epidemiological study particularly described the relation between using of specific ARGs and the presence of these genes in the environments. Also, presence of the ARGs and MGEs in the tested slaughterhouse wastewater with poor drainage of sewages and no treatment system for inactivation of DNA make this place as a hotspot for dissemination of ARGs to the environment. This considers as a potential risk factor for human and animal health in the area where the study had performed.

## CONCLUSIONS

Heavy use of antibiotics for treatment of animal diseases and as growth promotes lead to develop of resistance against many antibacterial agents. Environmental elements represent as a hotspot for emerging and spreading of ARGs. The current study evaluated the possible role of farm animal slaughterhouse wastes in dissemination of antibiotic resistance. Presence and quantity of genes encoding resistance to the widely used antibiotics in veterinary application was estimated in wastes using specific primers, PCR and qPCR. The results showed that slaughterhouse wastes have high concentrations of different ARGs that encoding to broad spectrum antibiotics. Also, MGEs were detected in the tested samples with high quantity per ml of the sample. Our study concluded that farm animal slaughterhouse is one of the important sources of ARGs and has a crucial role in dissemination of ARGs to the animal and human environment.

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## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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