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Approach to enhance antibiotics efficiency towards uropathogenic bacteria

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ABSTRACT

Aims: Urinary tract infection (UTI) is a common infection caused by many virulent bacteria. Multidrug resistance (MDR) by bacteria represents a major therapeutic challenge worldwide. MRD bacteria have different mechanisms to avoid antibiotics; one of them is horizontal gene transfer. Such genes, encoding antimicrobial resistance, are easily transferred from one bacterium to another. Magnesium and calcium chloride ($MgCl_2$ and $CaCl_2$) have an effect on the permeability of bacterial cell membrane. We aimed these chemical materials could increase the antibiotics efficiency on multidrug resistance bacteria. 250 UTI specimens were collected to isolate multidrug resistant bacteria. Depending on antibiotics resistance, we selected three species of virulent bacteria: *Escherichia coli*, *Staphylococcus aureus* and *Proteus mirabilis*. Then, we tested the effect of $MgCl_2$ and $CaCl_2$ on their antibiotics resistance.
Methodology and results: The results showed that percentage of *E. coli* in UTI infection is the highest (45%), while *Enterococcus faecalis* is the lowest (3%). The effect of $MgCl_2$ and $CaCl_2$ on bacterial antibiotics resistance has been tested using different types of antibiotics. The findings showed that $MgCl_2$ has significant effect to aid antibiotics against bacteria. In particular, nalidixic acid has shown more efficiency against *E. coli* and *S. aureus* but not *P. mirabilis.* Using different concentrations of $CaCl_2$ increased the efficiency of gentamycin, amoxicillin and trimethoprim against *S. aureus*, while has increased the efficiency of ampicillin and nalidixic acid against *E. coli*. However, $CaCl_2$ has no effect on the efficiency of antibiotics against *P. mirabilis.* In addition, $MgCl_2$, and $CaCl_2$ had no toxic effects in both T24 and 5637 urinary bladder cell lines. Finally, plasmids were isolated from these species to detect any antimicrobial resistance gene such as *qnr-A*.
Conclusion, significance and impact of study: MDR distribution in the worldwide was increased, we highly recommend the avoidance of the random antibiotic usages. The salts $CaCl_2$ and/or $MgCl_2$ can be used at specific concentration to enhance the antibiotics permeability and therefore to decrease the antibiotic resistance.

Keywords: Urinary tract infection (UTI), $MgCl_2$, $CaCl_2$, T24 and 5637 urinary bladder cell lines, *qnr-A*

INTRODUCTION

Urinary tract infection (UTI) is a very common case as the urinary system has a direct contact with the external environments (Andersson *et al.*, 2007; Mohsin, 2015). UTI is also the second most common infection worldwide. Every year about 150 million people are diagnosed with UTI (Gonzalez and Schaeffer, 1999). Many studies revealed that the percentage of adult women with UTI is higher than in men (Dwyer and O'Reilly, 2002; Al-Shamarti, 2010; Al-Shamarti and AL-Muhnna, 2011; Al-Shamarti *et al.*, 2018). Many microbial pathogens are involved in UTI (Sussman, 2002; Beetz, 2003; Al-Shamarti *et al*., 2018). The most common pathogen is *Escherichia coli*, and followed by *Proteus mirabilis*, *Staphylococcus saprophyticus*, and *Klebsiella pneumonia* (Mohsin, 2015). The causative agents of UTI are mostly bacteria (95%); however, fungi and viruses are also involved (Farajnia *et al.*, 2009; Khamees, 2012). Bacteriuria (bacterial colonization) is considered a major problem of cases as UTI (Bharti *et al*., 2019). A reliable diagnosis to determine the infection rather than the contamination rate is of utmost importance. Morgan *et al* have shown that the bacterial incidence in the urinary tract is around 10^5 CFU/mL (Morgan and McKenzie,1993). However, this threshold still holds in asymptomatic patients, in many cases, this is considered a sign for symptomatic patients.

The antibiotic resistance of UTI is changing over the last years, in both environmental and nosocomial

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infection. Different broad-spectrum antibiotics are often used as a UTI treatment when narrow-spectrum antibiotics are not appropriate as a treatment (Okeke *et al*., 2005). In recent years, the development of antibiotic resistance of antimicrobial agents had become a major problem worldwide; the reason might be the extensive antibiotic used (Okeke *et al*., 2005; Davies and Davies, 2010; Laxminarayan *et al*., 2013; Al-Shamarti and Mohsin, 2019). However, there are no many reports on the incidence of etiological agents and resistance pattern of acquired UTI in Iraq. Unsurprisingly, bacteria could express many resistance genes that is transferred and recruited from other epidemic strains. These resistance genes can encode the broad-spectrum beta-lactamases and aminoglycoside-blocking 18s RNA. Much of the beta-lactamases and aminoglycoside-blocking have been linked with epidemic potential strains, which are spreading across countries and continents, for example, *E. coli* with CTX-M-15 ESBL (Livermore, 2012). Interestingly, many researchers have observed that the antibiotic combination with organic or non-organic chemical materials increases antibiotics efficiency. Previous study by, Kligman (1965) and Garza-Ortiz *et al*. (2008) have demonstrated that some metal salts have positive effects on antibiotics efficiency. Furthermore, other studies have also suggested that the chemical metals has an effect on cellular enzymatic processes, this effect could promote the pharmacokinetic of tetracycline in the cell membrane (Upritchard *et al*., 2007; Bowman *et al*., 2011). In addition, some chemical compounds promote intestinal absorption such as phosphate compound, Mg^{2+}, $Mn^{2+,}$ Fe^{2+}, and Fe^{3+} and citric acid. These chemical materials could affect not only the efficiency of antibiotics but also affect the virulence factors (Laskin, 1967). Alsade *et al.* (2011) have reported that using inorganic phosphate promotes *Pseudomonas aeruginosa* growth. This compound has also affect on antibiotics resistance and virulence factors of *P. aeruginosa,* pyocyanin production*.* These observation leads us to perform a series of comparative experiments on the influence of magnesium and calcium ions on the intracellular accumulation of some antibiotic in both susceptible and resistant strains of UTI infection. This work is important for biologists and clinicians in order to facilitate and improve the treatment of UTI patients. Altogether, we planned to isolate different species from urinary tract infection, to demonstrate which has highest antibiotics resistance, and to explore the effect of $CaCl_2$ and $MgCl_2$ to increase the efficiency of some antibiotics against selected bacteria.

MATERIALS AND METHODS

Bacteria identification

Urine samples were collected using the mid-stream method to collect 250 urine samples from patients who have UTI infection. These samples were tested bacteriologically using standard procedures. In brief, a single colony was taken from each primary positive culture on blood agar and on MacConckey agar. Single colony was identified based on its morphology (colony shape, size and colour) and microscopical examination using the Gram's stain. The entire samples were taken and identified using VITEK2 microbial identification system in different hospitals in Iraq (Alsadar medical city and Alforat hospital in Alnajaf province and medical city in Baghdad province). For more validation, different biochemical tests were performed including: IMViC tests (Indole, Methyl red, Voges-Proskauer and Citrate tests), sugar fermented urease test, iron production, H_2S formation and certain enzyme production (data not shown).

Antibiotics sensitivity test

The Kirby-Bauer standardized single disk method was carried out following manufacture instructions (Bauer *et al*., 1966). Briefly, Mueller Hinton medium was prepared and autoclaved. Bacteria are evenly seeded throughout the plates using an L-shape sterile spreader. The bacterial cells density was approxiamatly1.5×10^8 cell/mL. This density was compared with 0.5 McFarland standard tube. Commercial antibiotics disks were used with a standard antibiotics concentration. These disks are evenly dispensed and lightly pressed onto the medium. All plates were incubated overnight at 37 °C in incubator. After incubation, the diameters of the complete inhibition zones were measured by using a ruler. Inhibition zone was measured to the nearest millimeter (mm) as compared to the zones of inhibition determined by National Committee for Clinical Laboratory Standards (NCCLS) (Ferraro, 2000).Therefore, to decide the susceptibility of bacteria to antimicrobial agent, whether being resistant or sensitive (Sader *et al*., 2003).

Effect of $MgCl_2$ and $CaCl_2$ to aid antibiotics against bacteria

Briefly, Mueller Hinton plates were prepared. Different concentration, which are 0.1, 0.05 and 0.025 µg/mL of $MgCl_2$ and $CaCl_2$ were prepared and added separately to the commercial antibiotics disks with a standard antibiotics concentration. The plates were incubated for 24 h to saturate all disks with $MgCl_2$ and $CaCl_2$. Finally, this experiment was preformed according to antibiotics sensitivity test (Bauer *et al*., 1966).

Culturing of urinary bladder cell lines

The conditions of incubation were 37 °C and 5% CO_2. Subculture of T24 and 5637 urinary bladder cell lines were carried out using trypsin. Briefly, the medium was discarded, and cells were washed using 5 mL Hanks' Balanced Salt Solution (HBSS). Then 2.5 mL of 1× Trypsin/EDTA (Lonza) was added and incubated for 20 min. 7.5 mL of McCoy's 5A medium modified containing serum was added and re-suspended by gentle aspiration for T24 cell line, while RPMI 1640 medium was used for

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5637 bladder cell line. Both cell pellets were centrifuged at 300× *g* for 5 min. 5 mL media was added to the pellet and re-suspended with complete media. Then cells were counted using a haemocytometer and an appropriate number of cells were used for seeding in a 96 flat bottom well plates.

Cytotoxicity assay- MTT

Both types of cells were seeded at 1×10^5 cell/mL in a flat bottomed 96-well plate in 100 µL complete media [McCoy's 5A or RPMI 1640 medium + 10% fetal bovine serum (FBS)] per well, and incubated at 37 °C, 8% CO_2 for 24 h. The media was gently aspirated, and cells were washed 2× with 100 µL HBSS. Different concentration of $MgCl_2$ and $CaCl_2$ were prepared (10 and 20 mg/mL) in suitable media + 1% FBS. The cells were treated with 100 µL of the indicated concentration from each with 4 replicate wells. There were also 2 controls: no treatment positive cell death (with 2% Triton X-100), and only media without cells. After incubation for 24 h at 37 °C, 8% CO_2, the media was removed, and washed with 2× HBSS. MTT (Sigma M5655) was prepared at 0.5 mg/mL in free medium without FBS, and 100 µL of free medium was added to each well. After 4 h incubation at 37 °C, 5% CO_2 in the dark, the media was gently aspirated and washed again with 1× HBSS. Finally, 100 µL DMSO was added, and left at room temperature for 1 h. LT-4000 microplate reader was used to measure absorbance at 570 nm.

PCR detection of quinolone-resistance gene encoded in plasmid (*qnr-A*)

DNA plasmid were extracted from all bacteria strains using QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. *qnr-A* gene was amplified by using primers *qnr-A* (5′-F TTTACCGCCAAATCGAACC; R 5′ CACCTTTTTGCTCCTCATCC) to produce a 183 bp product. Primer was ordered from Thermo Fisher Company. PCR conditions were amplified at 94 °C for 1 min, 61 °C for 30 sec, and 72 °C for 1 min. PCR product identity was confirmed by gel electrophoresis on a 1% agarose solved by Tris-EDTA buffer (Sigma, St. Louis, Mo.), and stained with 0.5 mg of Gel Red/mL and photographed under UV light. Species positive and negative for *qnr-A* were included in each batch of strains tested.

Gel electrophoresis

PCR product was separated based on their size, using gel electrophoresis. 1 g/mL of agarose from Fisher Scientific was dissolved in tris base, acetic acid and EDTA (TAE) buffer by boiling. The agarose solution was left in room temperature to allow cooling around 55 °C, and then 10 µL of Gel Red stain was added for each 200 µL of agarose gel to stain the DNA fragments. A suitable comb was placed into a gel cast before pouring for 30 min. The gel was transferred to an electrophoresis tank and the position of wells at the cathode end. Typically, 1 µL of 5× DNA gel loading dye (Thermo) and 5 µL of samples were mixed before loading in the gel. DNA ladder was loaded with a suitable volume recommended by the manufacturer Bioline®. Depending on the gel size, the gel was electrophoresed at a voltage (80-120) for 50-70 min. The UV transilluminator was used and images was captured using an EDAS 290 imaging system (Kodak) to visualize the DNA pieces.

Statistical analyses

Statistical analyses are performed using GraphPad Prism.8. Significance was determined using two-way ANOVA using Bonferroni post hoc analysis for multiple comparisons. Data are presented as mean ± standard error of mean (SEM).

RESULTS

Isolation of bacteria

The main aim was to investigate which species are the most common pathogens in UTI. We collected 250 specimens from different hospitals in Iraq. We started to identify bacteria form all samples depending on phenotyping, biochemical tests, morphology shape and selective media. Findings showed that only 136 specimens have bacteria, but other samples have no bacteria. The highest number is *E. coli* about 64 (45.5%) species. The lower number is *Enterobacter cloacae* around 5 (3.6%) species. However, other species included: *K. pneumoniae* percentage was 13.2%. *S. aureus* 16.9%, *P. mirabilis* 15.4%, and *P. aeruginosa* 5.14%, as demonstrated in Figure 1. It seems that the main causative agent is *E. coli*.

Antibiotics sensitivity test

As shown in Figure 1, *E. coli*, *P. mirabilis* and *S. aureus* percentages appeared to correlate closely with the onset of UTI. These three strains were selected to closely study. The aim of this section was to select the highest species to antibiotics resistance. The results showed that there is a variation of antibiotics resistance for isolates. All isolates were screened for antibiotics sensitivity and resistance (data not shown). *E. coli* (sample 40) *P. mirabilis* (sample 19) and *S. aureus* (sample 20) have highest antibiotics resistance than other samples, as demonstrated in Figure 2.

Effect of $MgCl_2$ and $CaCl_2$ on bacterial resistance towards antibiotics

The aim was to show if there is any effect of $MgCl_2$ and $CaCl_2$ on bacterial antibiotics resistance. The data shows that there is a significant effect on *E. coli* resistance after adding $MgCl_2$ as compared to control. The efficiency of ampicillin, gentamicin and nalidixic acid were significantly increased compared with control, as described in Figure 3A. In particular, there is a massive positive effect on

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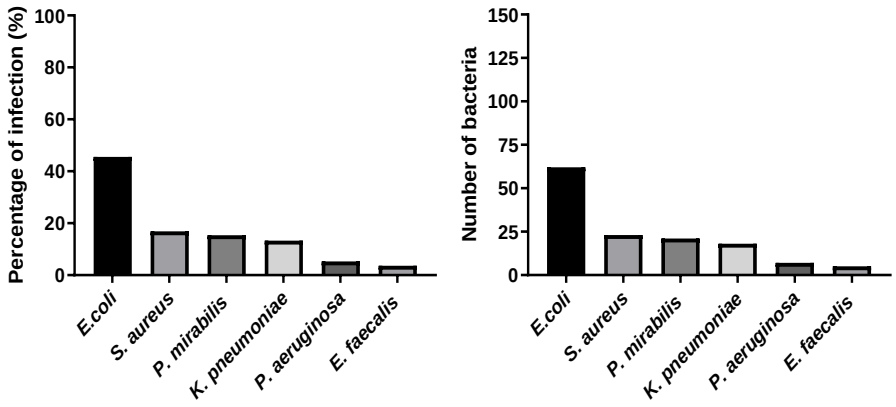

Figure 1: Percentage and number of different types of bacteria isolated from 250 samples of UTI infection from different hospitals in Iraq (Alsadar Medical city and Alforat hospital in Alnajaf province and Medical city in Baghdad province). Pure colonies were isolated and placed in VITEK2 microbial identification. For more validation, manual diagnostic methods have been performed which included biochemical and culturing tests.

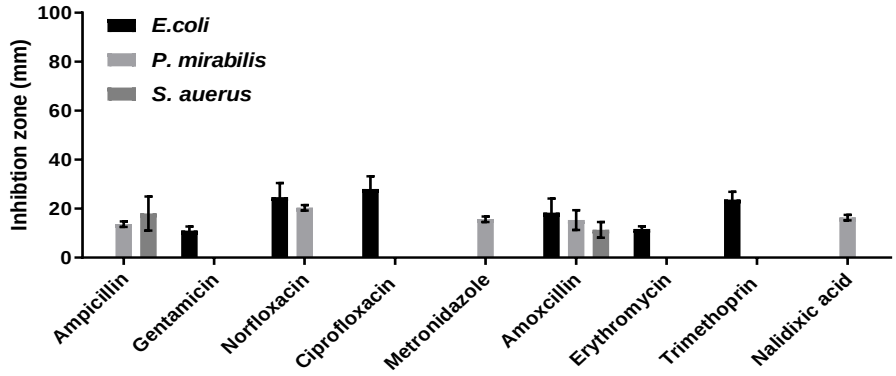

Figure 2: Antibiotic profiles of bacteria used in this study. All samples of bacteria are purified and plated on Muller Hinton agar using sterile swap, different types of antibiotics disk were placed on the plates, and bacteria were incubated for 24 h to measure the inhibition zone. The data are mean of 3 independent experiments with duplicates.

nalidixic acid efficiency against *S. aureus* and *E. coli* after adding $MgCl_2$ (Figure 3A and 3C). The findings also showed that there are positive effects of ciprofloxacin on *P. mirabilis* growth with different concentration of $MgCl_2$ compared with control group, as demonstrated in Figure 3B. Interestingly, *S. auerus* showed higher sensitivity to norfloxacin, nalidixic acid and trimethoprim after adding $MgCl_2$ compared with control (Figure 3C).

In addition, we used $CaCl_2$ to investigate its effect on bacterial antibiotics resistance. The data showed that there is also a huge effect of $CaCl_2$ on bacterial antibiotics resistance. As showed in Figure 4A and 4C, $CaCl_2$ increased effect of ampicillin and nalidixic acid on *E. coli*, while $CaCl_2$ has only effects of amoxicillin, gentamicin and trimethoprim efficiency on S. aureus growth compared with control group. However, $CaCl_2$ did not show any effect on *P. mirabilis* antibiotics resistance (Figure 4B).

PCR detection of *qnr-A* encoded in plasmid

The aim is to investigate the lack of *qnr-A* gene from selected isolates after treatment with $CaCl_2$. We did not test on $MgCl_2$ because it seems to have similar effect on antibiotics efficiency as $CaCl_2$, and it could be interesting work in the future with more details. The findings showed that *E. coli*, *P. mirabilis* and *S. aureus* have *qnr-A* gene in their plasmids, and some of bacteria could lose the plasmid, which encoded *qnr-A* gene after treatment with $CaCl_2$ at 0.1 µg/mL, as shown in Figure 5. It seems that $CaCl_2$ could have an effect on cell permeability of these pathogenic bacteria.

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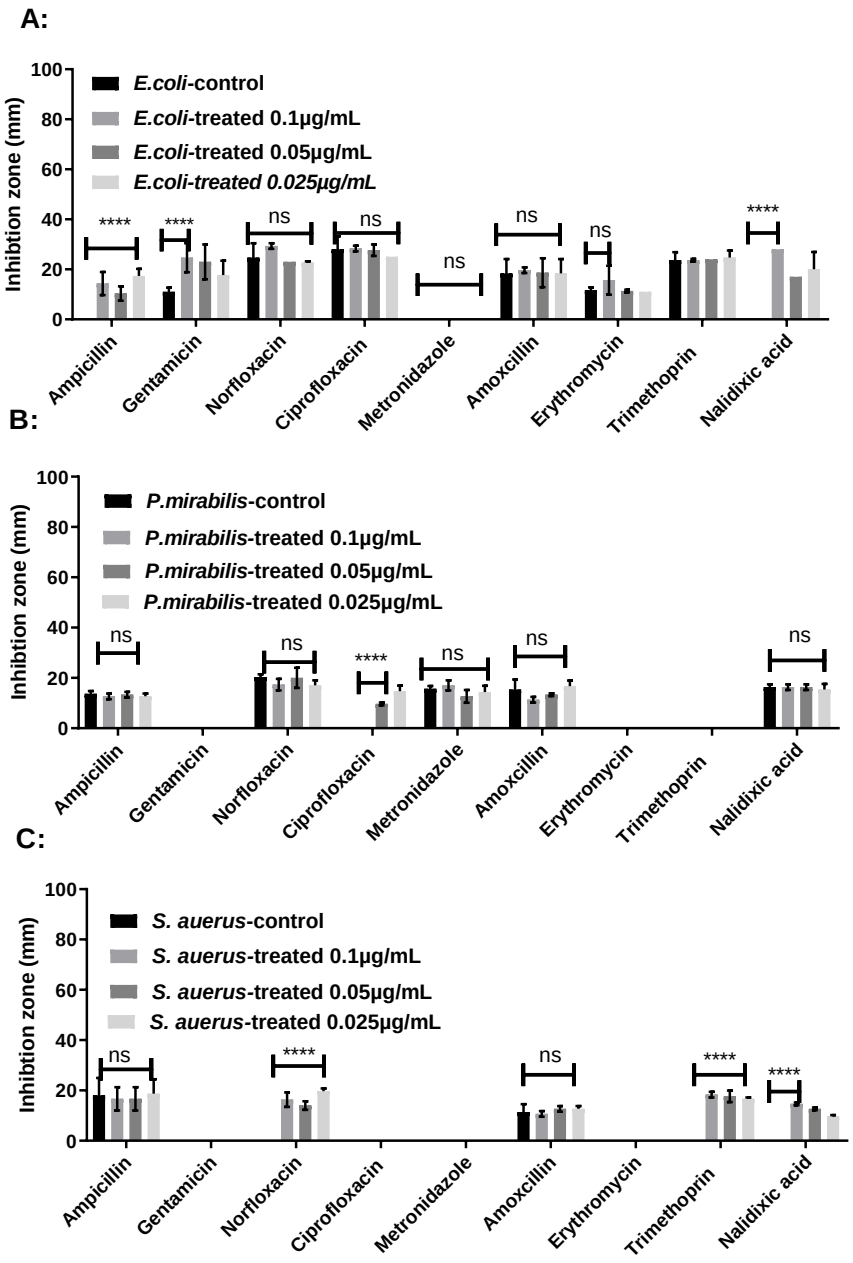

Figure 3: Effect of $MgCl_2$ on bacterial resistance of (A) *E. coli*, (B) *P. mirabilis* and (C) *S. aureus* towards antibiotics. Muller Hinton agar were prepared and different concentration of $MgCl_2$ (0.1, 0.05 and 0.025 µg/mL) was added to antibiotic disks. Different types of antibiotic disks with different concentration of $MgCl_2$ were placed on the plates, and bacteria were incubated for 24 h to measure the inhibition zone. The significance of the differences between treatments were tested by two-way ANOVA, where *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 significant; ns=non-significant. The results are the means of three separate experiments with duplicates.

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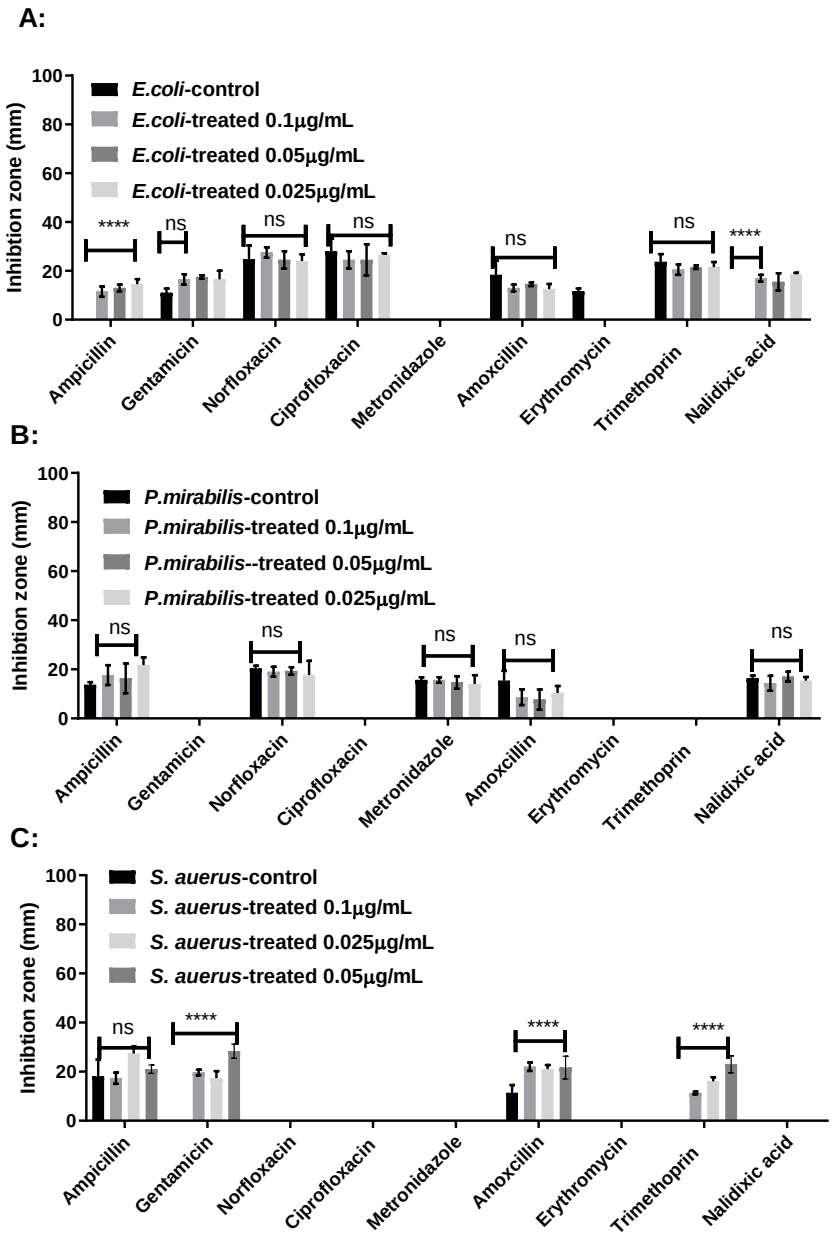

Figure 4: Effect of $CaCl_2$ on bacterial resistance of (A) *E. coli*, (B) *P. mirabilis* and (C) *S. aureus* towards antibiotics. Muller Hinton agar were prepared and different concentration of $CaCl_2$ (0.1, 0.05 and 0.025 µg/mL) was added to disks. Different types of antibiotic disks with different concentration of $CaCl_2$ were placed on the plates, and bacteria were incubated for 24 h to measure the inhibition zone. The significance of the differences between treatments were tested by two-way ANOVA, where *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 significant; ns=non-significant. The results are the means of 3 separate experiments with duplicates.

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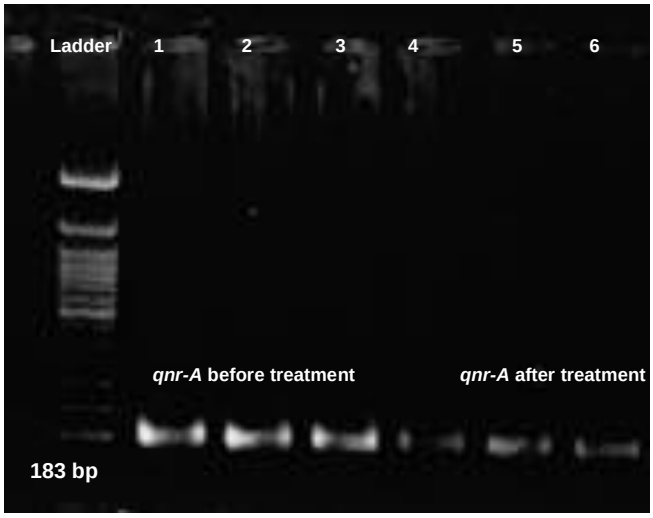

Figure 5: *qnr-A* gene encoded in plasmid before and after treatment with $CaCl_2$. Ladder lane: supercoiled DNA ladder (1000 bp). Lane 1: *qnr-A* in *E. coli* ; Lane 2: *qnr-A* in *P. mirabilis*; Lane 3: *qnr-A* in *S. aureus*; Lane 4: *qnr-A* in *E. coli* after treatment with $CaCl_2$; Lane 5: *qnr-A* in *P. mirabilis* after treatment with $CaCl_2$; Lane 6: *qnr-A* in *S. aureus* after treatment with $CaCl_2$.

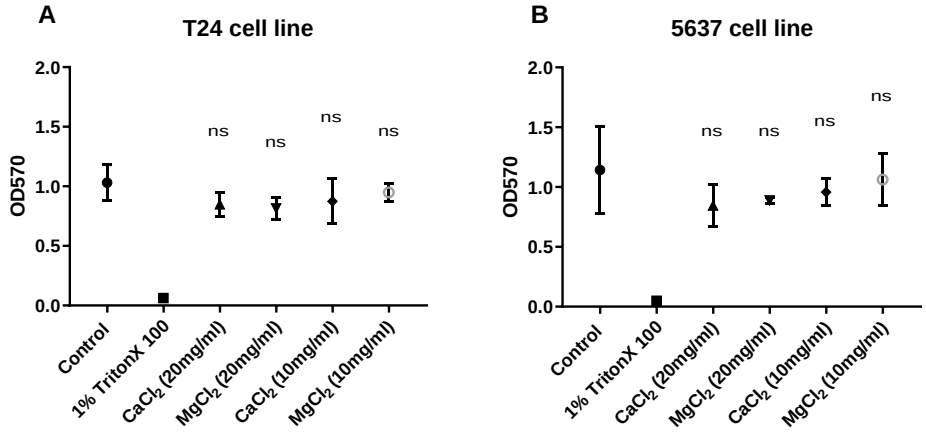

Figure 6: The effect of $CaCl_2$ and $MgCl_2$ on human cell lines. (A) T24 bladder cell line was treated with two different concentrations of $CaCl_2$ and $MgCl_2$ (10 mg/mL and 20 mg/mL). (B) 5637 bladder cell line treated with two different concentrations of $CaCl_2$ and $MgCl_2$ (10 mg/mL and 20 mg/mL). Control means untreated cells. The effects of different treatments were tested by unpaired t-test and compared to untreated cells, where ns=non-significant. The results are the means of 3 experiments with 8 replicates.

Cytotoxicity effect of $MgCl_2$ and $CaCl_2$ on human cell lines using MTT

This work was aimed to investigate the effects of $MgCl_2$ and $CaCl_2$ on mammalian cells number, and to detect early cytotoxic effect. In this study, two type of bladder cell lines were used: T24 and 5637 cell lines. The results show that both cells treated with $MgCl_2$ and $CaCl_2$ have similar OD_{570} compared to untreated mammalian cells. It was also noticed that increasing the incubation time had no toxicity (data not shown). It seems that $MgCl_2$ and $CaCl_2$ have no effect on cell density and no toxicity with T24 and 5637 cell lines with either concentration 20 mg/mL or 10 mg/mL, as demonstrated in Figure 6.

DISCUSSION

Our aims were to demonstrate the common pathogens in UTI, and to select highest bacterial antibiotics resistance. In addition, we attempt to find a novel strategy for enhancing the antibiotic activity against pathogenic bacteria. We found that *E. coli* is the main causative

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agent in UTI (Figure 1). This agree with the previous findings of many studies. For examples, Gunther *et al.* have found that *E. coli* percentage was about 88.2% and *K. pneumonia* was 66% (Gunther *et al*., 2001). Johnson and Russo (2002) also found *E. coli* is most common pathogens in UTI and other studies have been shown that the percentage of *E. coli* is about 40% (Johnson and Russo, 2002; Mehta *et al*., 2012; Mohsin, 2015). It has been revealed that *P. mirabilis* has a high resistance to ampicillin, trimethoprim and has a sensitivity to ciprofloxacin (Peterson *et al*., 2007). However, this does not support our findings. We revealed *P. mirabilis* has a sensitivity to ampicillin. Another study supported our result, which demonstrated *S. aureus* to have resistance to erythromycin and gentamicin (Figure 2) (Brown *et al*., 2012).

In the Figures 3 and 4, we investigated the role of $MgCl_2$ and $CaCl_2$ to increase the antibiotics efficiency. Interestingly, we showed that both chemical compounds were effective in decreasing bacterial antibiotics resistance. We also tested one of these chemical materials which is $CaCl_2$. We have shown that $CaCl_2$ could aid the bacteria to lose their plasmid DNA molecules that is responsible for antibiotics resistance (Figure 5). The explanation for that, these two chemical compounds can affect the cell membrane permeability of bacteria. Interestingly, pervious work has also explained that $CaCl_2$ makes bacterial cell wall negatively charged, and then reacts with the carbonate ions, which creates the calcium carbonate on the bacteria cell surface (Ksara *et al*., 2019). This could explain how these compounds increase the potency of antibiotics against different bacteria. Furthermore, $MgCl_2$ and $CaCl_2$ are involved in creation pores in the cell membrane of bacteria (Green and Sambrook, 2018). This is another reason explaining how $CaCl_2$ and $MgCl_2$ aid antibiotics against bacteria. Another study, Berlanga *et al.* used carbonyl cyanide *m*-chlorophenyl-hydrazone to Muller Hinton media to investigate its effect on ciprofloxacin efficiency against *E. coli* and *S. aureus.* They found that there is a significant increase in ciprofloxacin efficiency after adding this chemical factors (Berlanga *et al*., 2004). It is probably that the pharmacokinetic of ciprofloxacin needs some extra chemicals compounds. We also found, bacterial strains isolated from UTI could lose their plasmid after treatment with $MgCl_2$ and $CaCl_2$. That could also explain the effect of $MgCl_2$ and $CaCl_2$ on bacterial resistance. This findings supported by Liu and his team who demonstrated that $CaCl_2$ can reduce the efficiency of transformation using competency induction method (Liu *et al*., 2014). Allard and Bertrand have explained that tetracycline resistance could be lost in *E. coli* transformed with plasmid pBR322 after treatment with silver nanoparticles (Allard and Bertrand, 1992). This supports our suggestion related to $CaCl_2$ and $MgCl_2$, that there is a significant increase in cell permeability, which leads to remove bacterial plasmids of MDR. As shown in Figure 6, our study also revealed that there is no significant differences between the two cell lines concerning their sensitivity to $CaCl_2$ and $MgCl_2$ with both concentration 10 mg/mL and 20 mg/mL. There are limited number of publications that explain the effect of some chemical materials on cell viability, however no publications in regard to $CaCl_2$ and $MgCl_2$. Fotakis and Timbrell (2006) have shown that cadmium chloride ($CdCl_2$) has no effect on HepG2 and HTC cells. They also found that the cell membrane could be rupture when lower concentrations of cadmium chloride or shorter incubation times in both host cells.

CONCLUSION

It is, thus, likely that bacterial mechanism of antibiotics resistant continue to occur but the records are not currently updated in Iraq because of clinician staff in many hospitals have a lack of awareness of UTI and underdeveloped diagnostic microbiology services. We recommend that antibiotic random use must be avoided; otherwise, the problem of MDR will be increased. This study proposed $CaCl_2$ and $MgCl_2$ could be added to antibiotics as a new therapeutic approach to overcome the bacterial antibiotics- resistance problem.

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