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# Effect of Some Metals Ions on Hemolysin Production from Clinical Isolates of *Escherichia coli*

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**Abstract**

**Objective:** This study aimed to determine effect of some ions metals ($Ca^{+2}$, $Mn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$, $Zn^{+2}$) on hemolysin production was performed.

**Methods:** Eighty Three (83) *E. coli* isolates collected from different clinical sources from Baghdad Hospitals, performed by using cultural traits, morphological features and biochemical tests, confirmation of identification was done by Vitek 2 system. And it was detected on ability these isolates to production hemolysin by two methods liquid media and agar media.

**Results:** The results showed that: In agar medium revealed that 6 (7.23%) isolates of *E. coli* had the ability for producing this enzyme and 77 (92.77%) did not produce hemolysin. The same results were found in liquid medium. Used the microtitration plate method to study the effect these ions metals on hemolysin production. According to the effect of ions metals, the results also showed that MIC value of ions metals was 500 µg/mL, hemolysin production was increased after addition each of ($Ca^{+2}$, $Mn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$) the percentage of hemolysis (96.0%, 93.4%, 93.2%, 93.3%, 94.2%, 93.7%) respectively while hemolysin production decreased after addition of $Zn^{+2}$ with the percentage of hemolysis 0.3%.

**Conclusion:** Based on the influence of metal ions, the findings indicated that hemolysin production exhibited an increase following the addition of each of $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $K^{+}$, and $Na^{+}$ when compared to the control group. Conversely, the introduction of $Zn^{2+}$ led to a decrease in hemolysin production.

**Keywords:** *Escherichia coli,* hemolysin, metals Ions, MIC, microdilution method

## Introduction

*Escherichia coli* are a highly researched bacterium that is widely regarded as one of the most completely studied model microorganisms. It acts as a commensal organism within the gastrointestinal tract, being among the initial bacterial species to establish colonization shortly after birth. Additionally, it remains significant relevance as a pathogen, capable of inducing both intestinal and extra-intestinal infections in humans and animals.[1] Hemolysins have been found as significant virulence factors in a range of bacterial infections, *E. coli* hemolysins may be classified into three distinct categories: namely coli α-hemolysin (HlyA), enterohemolysin (EhxA), and hemolysin E (HlyE) (Murase *et al*., 2012; Abid *et al*., 2022).[2,3]

Pathogens often suffer from metal limits throughout the course of an illness. Changes in the levels of certain metal ions have a major effect on cellular physiology and the regulation of gene expression. For example, changes in the iron concentration have a role in controlling the activation of virulence factors by means of metalloregulatory proteins like Fur; which is the ferric uptake regulator; and its closely related counterpart PerR; which is the peroxide-stress regulator.[4] Various factors can influence the production of hemolysin, including the presence of limited metals, which is frequently observed during infection and may have a major effect on the pathogen's viability. For instance, a reduced concentration of the toxin triggers an elevation in intracellular calcium levels, leading to the activation of calpains that degrade cytoskeleton proteins. Additionally, this condition promotes the externalization of phosphatidylserine (PS) on the outer surface of erythrocyte membranes.[5]

The aim of this study was to evaluate the effect of some ions metals for hemolysin production from *E. coli* bacteria which was isolated from different clinical sources.

## Materials and Methods

### Bacterial Isolates

Total of 83 clinical isolates were obtained from various clinical sources, including urine, stool, wound, and blood, from hospitals in Baghdad. The isolates were sub-cultured on MacConkey agar, Blood agar, and Nutrient agar. The determination of phenotypic features was conducted based on the colony morphology seen on culture medium, which exhibited variations in form, size, odor, color, texture, opacity, and margin of colonies. The Gram stain technique was used to identify and distinguish the morphology and organization of bacteria via microscopic analysis.[6] The confirmation of all isolates was conducted using the VITEK 2 technology.

### Screening of E. coli for Hemolysin Production

#### *Plate method (agar medium)*

Bacterial suspensions were prepared in sterile saline solution with a concentration of $1.5 \times 10^8$ CFU/ml, derived from 18-hour cultures of *Escherichia coli* isolates. A volume of 10 µl from each suspension was cultured onto the surface of the blood agar medium, followed by incubation at a temperature of 37°C for duration of 16 hours. The examination of hemolysis was conducted after duration of 16 hours, as reported by Shimuta et al.[7]

#### *Spectrophotometric method (liquid medium)*

The spectrophotometric approach published by Di Venanzio et al.[8] with certain modifications was used, specifically in *E. coli* bacteria were cultured overnight in a nutrient broth at a temperature of 37°C. Then centrifugation at 3000 rpm for 10 minutes. Red blood cells (RBCs) was suspended in

0.8 ml of PBS buffer. To this suspension, 9.2 mL of PBS buffer was added, resulting in a final volume of 10 mL. separately, 1 ml of a bacterial supernatant was added to 1 ml of the RBC suspension. The mixture was then incubated at 37°C for 1 hour. The existence of hemolysin was assessed by conducting centrifugation at 12,000 revolutions per minute for duration of 10 minutes. Subsequently, the measurement of hemolysin production was performed using a spectrophotometer set at a wavelength of 571 nm. Hertle *et al* (1999)[9] established that the percentage of hemolysis was determined by calculating the relative optical density (OD) of a sample in comparison to the OD of complete hemolysis of red blood cells (RBCs), which was achieved by introducing 1% Triton X-100.

Calculation of hemolysis percent:

$$Hemolysis\% = \frac{\text{A571 (sample with hemolysin)} - \text{A571 (control without hemolysin)}}{\text{A571 (total lysis caused by Triton x-100)} - \text{A571 (control without hemolysin)}} \times 100$$

### Determination of the Minimum Inhibitory Concentration (MIC) for Some Ions Metals on Growth of E. coli

The antibacterial activity of several ions metal ($Zn^{+}$, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$) was evaluated by the microdilution method. A solution including zinc sulfate ($ZnSO_4$), calcium sulfate ($CaSO_4$), magnesium sulfate ($MgSO_4$), manganese sulfate ($MnSO_4$), iron sulfate ($FeSO_4$), potassium chloride (KCl), and sodium chloride (NaCl) was prepared to get concentrations ranging from 1000 to 0.976 micrograms per milliliter ( µg/ml). A volume of 100 µl of each of the metals was individually combined with 100 µl of BHI broth and distributed into the wells of Column 1. Columns 2–11 were filled with 100 µl of BHI broth alone. Column 12 contained 200 µl of a diluted standardized inoculum, while Column 11 contained 200 µl of the medium broth. The *E.coli* culture ($1.5 \times 10^8$) using a 1:100 ratio with BHI broth. then, 100 µl of the adjusted bacterial suspension was applied to each well. The microplates were securely sealed and subjected to incubation at a temperature of 37°C for duration of 24 hours. Subsequently, a solution of resazurin dye at a concentration of 0.015% was introduced into each well, with a volume of 60 µl per well. The plate was then incubated for an additional 4 hours to allow for the observation of any changes in color. Following the incubation period, the column was without any observable change in color.[10]

### Effect of Some Ions Metals on Hemolysin Production from E. coli

The effects of metal ions ($Zn^{+2}$, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$) on the synthesis of hemolysin from *Escherichia coli,* the isolates of *E. coli* were cultivated in the absence and presence of various metal ions ($Zn^{+2}$, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$) at a subminimum inhibitory concentration (MIC) of 500 µg/ml. Then incubated at 37°C for 24 h. Then centrifuged at 10,000 rpm for 10 minutes. From each tube, only 1 ml was extracted and combined with 1 ml of bloodcell, followed by incubation at 37°C for one hour. Then OD was measured at 571 nanometers. Then the percentage of hemolysis production was estimated according to Hertle et al.[9]

## Results

### Isolation and Identification of E. coli

The colonies on MacConkey agar appeared pink in color and displayed a green metallic sheen when observed under the electron microscope (Figure 1).

### Microscopic Examination

The *Escherichia coli* isolates were observed under a light compound microscope, revealing their distinctive features as Gram-negative bacteria (Figure 2).

### Screening of E. coli Isolates for Hemolysin Production

Every *E. coli* isolate underwent testing for hemolysin production using the plate method. Table 1 presents the results of hemolysin production by clinical *E. coli* isolates using both plate and liquid medium methods.

### Determination of the Minimum Inhibitory Concentration for Some Ions Metals on Growth of E. coli

Several metal ions were employed in the study, including $Zn^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $K^{+}$, and $Na^{+}$. The Minimum Inhibitory Concentrations (MICs) of these metal ions were assessed using the modified broth micro-dilution method, as illustrated in Figure 3. The findings revealed that the MIC value for these metal ions was 500 µg/mL.

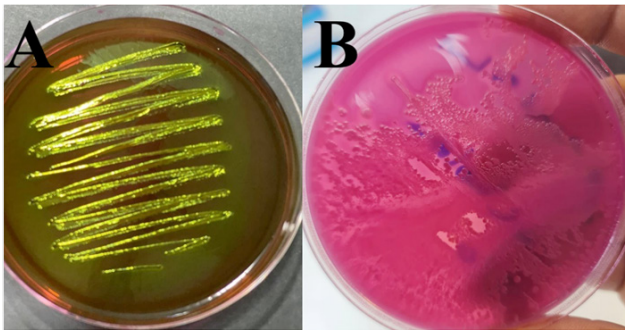


Fig. 1 ***E. coli* colonies on A: EMB agar and B: MacConkey agar.**

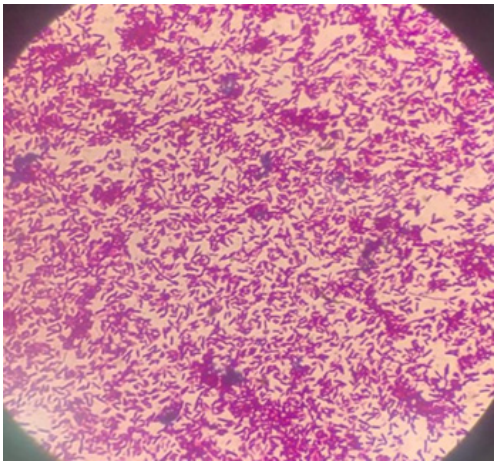

Fig. 2 ***E. coli* bacteria under light compound microscope.**

Table 1. **Hemolysin production by plate and liquid medium methods by the clinical *E. coli* Isolates**

| *E. coli* isolates No. | Clinical source | Plate method | Liquid medium-method |
|---|---|---|---|
| | | Diameter of hemolytic zone (mm) | Absorbance of hemolysin (OD-571 nm) |
| Ec 1 | UTI | 25 | 0.998 |
| Ec 2 | Stool | 16 | 0.925 |
| Ec 3 | Wound | 15 | 0.724 |
| Ec 4 | Blood | 13 | 0.513 |
| Ec 5 | Stool | 12 | 0.492 |
| Ec 6 | Wound | 10 | 0.322 |

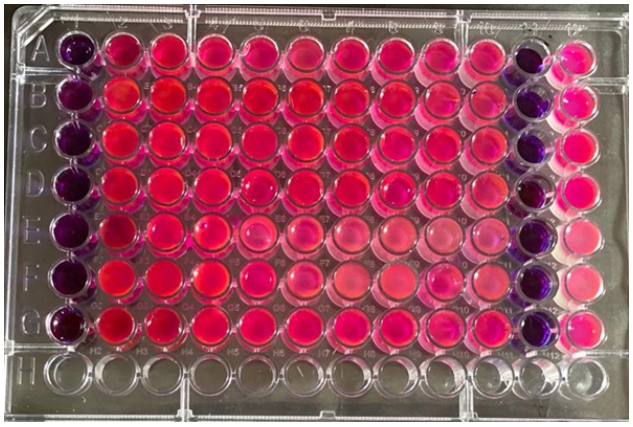

Fig. 3 **Microdilution method of some metals against clinical isolate of *E. coli*.**

### *Effect of Some Ions Metals on Hemolysin Production from Clinical Isolate of E. coli*

Table 2 shows the evaluation and the influence of metal ions on hemolysin production in a clinical isolate of *E. coli*. Specifically, we utilized ($Ca^{++}$) in the form of Calcium Sulfate ($CaSO_4$), ($Zn^{++}$) as Zinc Sulfate ($ZnSO_4$), ($Mn^{++}$) as Manganese Sulfate ($MnSO_4$), ($Mg^{++}$) as Magnesium Sulfate ($MgSO_4$), ($Na^{+}$) as Sodium Chloride (NaCl), ($K^{+}$) as Potassium Chloride (KCl), and ($Fe^{++}$) ions.

Table 2. **Effect of ions metals on hemolysin production from clinical isolate of *E. coli***

| Ions metals | Absorbance of hemolysin (OD-571 nm) | Hemolysis (%) |
|---|---|---|
| Control by triton X-100 | 3.0 | 100 |
| Control without treatment | 2.743 | 91.4 |
| $Zn^{+2}$ | 0.119 | 0.3 |
| $Ca^{+2}$ | 2.882 | 96.0 |
| $Mn^{+2}$ | 2.804 | 93.4 |
| $Mg^{+2}$ | 2.828 | 94.2 |
| $Fe^{+2}$ | 2.801 | 93.3 |
| $K^{+}$ | 2.828 | 94.2 |
| $Na^{+}$ | 2.813 | 93.7 |

## Discussion

### *Isolation and Identification of E. coli*

Based on clinical samples, *E. coli* has been identified. Based on morphological analysis use of Blood agar, MacConkey agar, and Eosin Methylene Blue (EMB). The observed colonies exhibited circular morphology with a convex shape. The surface of the colonies appeared smooth upon initial isolation. On MacConkey agar, the colonies displayed a pink coloration. The growth of *Escherichia coli* is characterized by the presence of a green metallic sheen, which may be related to the metachromatic property of dyes.[11]

### *Microscopic Examination*

The *Escherichia coli* isolates were seen using a light compound microscope, showing their characteristics as Gram-negative bacteria. They showed a rod-shaped morphology and were found to be organized either alone or in pairs.

### *Screening of E. coli Isolates for Hemolysin Production*

All *E. coli* isolates were tested for production of hemolysin in blood agar. The results demonstrated the appearance of clear zones of hemolysis after the end of incubation period around the growing colonies with different diameters. According to hemolysin production 6 (7.23%) of (83) *E. coli* isolates had the ability for producing hemolysin and 77 (92.77%) were non-producers. The same results were found in liquid medium, as shown in Table 1. The results on Table 1 were closed to what Vaish *et al.* (2016)[12] concluded, they demonstrated that approximately 9% of the *E. coli* strains were capable of producing hemolysin. In a recent local research conducted by Ghaddar et al., 2020,[13] findings revealed the presence of Haemolysins in *E. coli* isolates were discovered in 52.2% of the samples. Similarly, a research performed in India reported a prevalence rate of 51.1% for *E. coli* isolates. Salman *et al.*, (2013)[14] found that the presence of haemolysis of UTI-*E. coli* isolates on blood agar was observed in 6 (22.2%) of total isolates, in contrast the present results non of urine isolated isolates exhibited hemolysin production, in contrast to Abdel Rahman, (2006)[15] who revealed that 70.5% of *E. coli* isolated from chronic UTIs were hemolytic.

### *Determination of the Minimum Inhibitory Concentration for Some Ions Metals on Growth of E. coli*

Some ions metals were used in the study: ($Zn^{+2}$, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$). The Minimum Inhibitory Concentrations (MICs) of these metals were determined by the modified broth micro-dilution method (Figure 3). The results showed that MIC value of ions metals was 500 µg/mL. Metal ions have potent antibacterial properties; nevertheless, they may also induce cytotoxicity or reflect poor cytocompatibility.[16] In a study performed by Heidenau *et al.* (2005),[17] the antimicrobial activity of several metal ions against *S. epidermis* was investigated by using the plate count technique, and showed that $Hg_{+}$, $Ag_{+}$, and $Cu_{2+}$ have strong antibacterial activity, but

$Co_{2+}$ and $Zn_{2+}$ did not display a statistically significant antibacterial impact at the quantities examined. Du *et al.* (2009)[18] found that the MIC of $Mn_{2+}$ against *E.coli* was measured to be 26812.3 µM and MIC of $Mn_{2+}$ was 29143.9 µM against *Staphylococcus*, also the same study demonstrated that the minimum inhibitory concentration (MIC) of $Zn_{2+}$ against *Escherichia coli* and *Staphylococcus aureus* was determined to be 11743 µM.

The strong antibacterial activity of metals may be attributed to the formation of metal ions, leading to a correspondingly elevated concentration of metal ions and, thus, an increased antibacterial effect. However, it is important to note that the elevated concentration of metal ions may also result in cellular damage. Based on the factors mentioned above, the most optimal selection for the development of antibacterial metals would include alloying elements characterized by low minimum inhibitory concentration (MIC) and high lethal dose 50 (LD50). Major examples of such elements are $Fe^{3+}$, $Ag^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$. The antibacterial properties of $Ag^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ have been extensively documented in studies; however the antibacterial potential of $Fe_{3+}$ and $Co_{2+}$ remains unknown according to research by Zhang et al.[16]

### *Effect of Some Ions Metals on Hemolysin Production from Clinical Isolate of E. coli*

In the current study, the effect of ions metals was examined on hemolysin production from clinical isolate of *E. coli* by using ($Ca^{++}$) as Calcium Sulfate ($CaSO_4$), ($Zn^{++}$) as Zinc Sulfate ($ZnSO_4$), ($Mn^{++}$) as Manganese Sulfate ($MnSO_4$), ($Mg^{++}$) as Magnesium Sulfate ($MgSO_4$), ($Na^{+}$) as (NaCl), ($K^{+}$) as (KCl) and ($Fe^{++}$), the results showed that hemolysin production was increased after addition (at sub MIC concentration 500 µg/ml) each of of ($Ca^{+2}$, $Mn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, $K^{+}$, $Na^{+}$) with percentage of hemolysis (96.0%, 93.4%, 93.2%, 93.3, 94.2%, 93.7%) respectively while hemolysin production decreased after addition of $Zn^{+2}$ with the percentage of hemolysis 0.3%, as shown in Table 2. The results of this study agreed with the research conducted by Bücker *et al.* (2020),[19] which identified using of zinc as a therapeutic option.

Bücker *et al.* (2020)[19] also investigated the basic mechanisms of epithelial barrier dysfunction induced by *Escherichia coli* expressing alpha-hemolysin (HlyA) in the colon mucosa, as well as the potential reducing effects of zinc ions, the authors indicated further investigations on the potential impact of zinc's inhibitory mechanism on the process of HlyA pore formation. Hemolysin inhibition may be seen in the presence of several cations, including zinc, barium, and lanthanum. The study conducted by Wiegand *et al.* (2017)[20] shown that zinc functions as an inhibitor, specifically targeting the HlyA-induced calcium influx and cause leak development in the colon epithelium.

Furthermore, the present study supports the findings of Velasco *et al.* (2018),[21] who demonstrated that zinc possesses a defensive impact against α-hemolysin-induced damage. Notably, the data presented by Velasco *et al.* (2018)[21] suggest that zinc could provide an extra protective effect by facilitating the downregulation of α-hemolysin expression.

In a study by Caetano *et al.* (2022),[22] they investigated the functional characteristics of the hemolytic activity uropathogenic *E. coli* that produce alpha-hemolysin was first assessed using blood agar, with and without the addition of $CaCl_2$, the results of their study clearly shown that the presence of $CaCl_2$ is necessary for hemolysin to induce hemolysis in blood agar. Conversely, in the absence of $CaCl_2$, hemolysis did not occur.

In a study done by Zhen *et al.* (2015),[23] the researchers investigated the effects of pure $Mg^{2+}$ on blood hemolysis without using bacteria, the study results indicated that as the concentration of $Mg^{2+}$ increased, the ratio of hemolysis also increased. This observation suggests that elevated levels of $Mg^{2+}$ could lead to increased osmotic pressure on blood cells. Consequently, these results may indicate the potential presence of hemolysin-produced-*E. coli* with addition of Mg salts may potentially increase or enhance the process of blood hemolysis on blood agar.[22]

In a study by Johnsen *et al.* (2019),[24] it was discovered that *E. coli* strains showed noticeably bigger colonies when cultivated on blood agar plates, so providing evidence for the potential of enhanced iron availability to facilitate the proliferation of *E. coli*.

Sedat (2020)[25] performed a research investigating the expression level of the hemolysin-encoding gene (*vvhA*) and the growth rate of several strains of *Vibrio vulnificus* bacteria at varying iron concentrations. Notable difference was observed between the 30 and 50 µM ferric chloride concentrations in comparison to the lower concentrations across all tested strains. It was demonstrated that elevated levels of ferric chloride positively influenced the growth rates of the strains, indicating that an excess of iron serves as an inducer for bacterial growth. Iron has a crucial role as a redox catalyst in a multitude of biological functions, including respiration and DNA replication, particularly in microbes.[26]

The data presented by Mouriño *et al.* (1998)[27] provided evidence about the presence of *E. coli* cells exhibit elevated synthesis of the Hha protein only in a medium characterized by high levels of NaCl are consistent with the findings that this protein modulates hemolysin expression in response to osmolarity.

According to the findings of Aldick *et al.* (2009),[28] it was determined that the hemolytic activity showed a major reliance on the presence of calcium. This observation may provide an explanation for the major effect of calcium on hemolysin production.

According to Do and Gries (2021),[29] pathogenic bacteria depend on potassium transport to satisfy their nutritional and chemiosmotic requirements. Additionally, potassium has been observed to directly influence the expression of virulence genes, antimicrobial resistance, and the formation of biofilms. Abdulkarim *et al.* (2009)[30] found that the growth of *E. coli* was slightly reduced when NaCl was absent from the medium. The *Escherichia coli* bacteria were incubated at a temperature of 37°C. The optimum growth of *Escherichia coli* occurred at a NaCl concentration of 0.5% (w/v). The addition of a 0.5% potassium chloride (KCl) solution was shown to have little effect on the proliferation of *E. coli* bacteria. Addition of 0.5% KCl was found to have little benefit on the growth of *E. coli* at 37°C compared to cells grown in medium with 0.5% NaCl, this may reflects the effect of KCl on hemolysin production.

## Conclusions

- ❖ According to the effect of ions metals, the results showed that hemolysin production was increased after addition

each of $Ca^{+2}$, $Mn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, $K^{+}$ and $Na^{+}$ compare to control.

- ❖ Hemolysin production was decreased after addition $Zn^{+2}$.

## List of Abbreviations

| | |
|---|---|
| Na: | Sodium |
| Zn: | Zinc |
| Mg: | Magnesium |
| K: | Potassium |
| Ca: | Calcium |
| Mn: | Manganese |
| Fe: | Iron |
| Cu: | Copper |
| Ag: | Silver |
| Co: | Cobalt |
| *E. coli*: | *Escherichia coli* |

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## Declaration of Interest

No conflict of interest.

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