

# *In-vitro* and *in-vivo* therapeutic study the effect of Ca-EDTA against *Salmonella typhimurium* infection in experimental local rabbits

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## Abstract:

This research was conducted for the purpose of evaluating the therapeutic impact of gentamicin and Ca-EDTA both alone and in combination. A highly virulent isolates of *S. typhimurium* were obtained from the department of internal and preventive medicine/ College of Veterinary Medicine/ University of Baghdad. A twenty five local rabbits of both genders with age range (2-4 months) weeks old were used for this study, the rabbits were divided randomly into five groups each group contains 5 rabbits :- group 1: drenched orally with 5 ml of normal saline and consider as control group, group 2: were drenched orally with (5 ml) suspension which contain ( $5 \times 10^9$  CFU) of *Salmonella typhimurium* and regarded as infected group, group 3 were drenched orally with (5 ml) suspension which have ( $5 \times 10^9$  CFU) of *Salmonella typhimurium* then treated with a single dose of gentamicin alone at 0.05ml/kg (5mg/ml) orally after presence of signs (after 24hrs. post inoculation), group 4 were drenched (5 ml) suspension having ( $5 \times 10^9$  CFU) of *Salmonella typhimurium* then treated with a single dose of Ca-EDTA alone at 40mg/kg orally after presence of signs (after 24hrs. post inoculation) and group 5 were drenched (5 ml) suspension that contain ( $5 \times 10^9$  CFU) of *Salmonella typhimurium* then treated with a single dose of combined gentamicin at 0.05ml/kg (5mg/ml) orally after presence of signs (after 24hrs. post inoculation) and Ca-EDTA 40mg/kg after presence of signs (after 24hrs. post inoculation).

The results of invitro activity showed a synergistic effect with using antimicrobial plus Ca-EDTA on agar plate and tube containing gentamicine and Ca-EDTA.

The results of invivo study showed a clear response to a combination therapy (Gentamicin + Ca-EDTA).

In conclusion, Ca-EDTA can act synergistically with gentamicin to treat the rabbits with salmonellosis by reducing length and frequency of treatment.

**Key words:** Salmonella, gentamicin, Ca-EDTA, rabbits.

## INTRODUCTION:

Salmonella can be defined as significant zoonotic pathogens that have an impact on the health animals and people all over the world. The classification system for organisms was improved due to the advanced techniques for detecting Salmonella and the improved knowledge regarding their genomics. Presently, genus Salmonella contain just two species, *S. enterica* and *S. bongori*, with the first having six sub-species: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI) (1).

As antibiotics are used widely, it has resulted in the up growth of various antibiotic-resistant bacteria such as *Salmonella typhimurium*. The different results of resistance may be due to the use of non-specific antibiotic and random administration of antibiotic and this will make persistent strains have ability to resist a large number of antibiotics (2). This issue has been reported to the concerned government and industry agencies state the increasing need for affective vaccines for handling this serious zoonotic infection (3).

Gentamicin is highly recommended antibiotic by many researchers in treatment of salmonellosis (4,5,6).

Ethylenediaminetetraacetic acid, which can be abbreviated to (EDTA) can be defined as a recognized metal-chelating agent, it is widely applied for treating patients who were subjected to poisoning with heavy-metal ions like lead and mercury (7). Large number of EDTA based antiseptic solutions usually have one or more EDTA salt in solution with efficiency specefied through pH of the treated environment. Sodium salts of the EDTA usually applied as antiseptic or as antibiofilm agents contain tetrasodium, trisodium, and disodium salts. Nevertheless, other salts related to the EDTA, such as ferric sodium, magnesium disodium, cupric disodium, di-potassium, potassium, di-ammonium, ammonium

and other combinations proved to have antibiofilm and antimicrobial abilities (8).

There are some intrinsic antimicrobial activities related to EDTA, such as neutralizing of bacterial enzymes/toxins, detoxication, potentiating the activity of other classes of antibiotics, and include direct antimicrobial effects. One of the most familiar types of action regarding EDTA is that does potentiate other antibiotics through the disruption of lipopolysaccharide structure in the outer membrane of Gram-negative bacteria. As a result of this disruption process, the membrane become more permeable to other agents (9,10).

This study was aimed to invitro and invivo evaluation of the therapeutic effect of Ca-EDTA alone and in combination with gentamycin on infected animals with *S. typhimurium*.

## MATERIALS AND METHODS:

A highly virulent isolate of *S. typhimurium* from domestic and street diarrheic and non-diarrheic dogs in Baghdad Province which obtained from Department of internal and preventive medicine/ College of Veterinary Medicine/University of Baghdad were previously diagnosed and confirmed by PCR as mentioned in previous study (11). Infective dose of bacteria was prepared as described by Miles and Misra, (12) containing  $5 \times 10^9$  CFU as recommended by Habasha *et al.*, (13).

The experimental study was conducted on 25 local rabbits of both genders aged between 2-4 months old were adapted for two weeks before starting the experiment by rearing in clean and disinfected cages, fecal cultures for *S. typhimurium* were done before infection to exclude the possibility of natural infection and carrier animals. They were fed on ad libitum alpha alpha and clean water. These rabbits divided randomly into five groups as follows:  
**Group 1** : five rabbits were used as negative control which

drenched orally by plastic syringe with normal saline at dose 5 ml, **Group 2**: five rabbits were used as infected group which drenched 5 ml suspension which have  $(5 \times 10^9)$  CFU of *Salmonella typhimurium*, **Group 3** : five rabbits were drenched 5 ml suspension having  $(5 \times 10^9)$  CFU of *Salmonella typhimurium* then treated with single dose of gentamicin (Vapco, Jordan) alone at 0.05ml/kg (5mg/ml) orally (according to manufacturer) after presence of signs (24hrs. post infection), **Group 4** : five rabbits were drenched 5 ml suspension containing  $(5 \times 10^9)$  CFU of *S. typhimurium* then treated with single dose of Ca-EDTA (GLS company, USA) alone at 40mg/kg orally (according to manufacturer) after presence of signs (24hrs. post infection), **Group 5** : five rabbits were drenched 5 ml suspension containing  $(5 \times 10^9)$  CFU of *S. typhimurium* then treated with combination of single dose of gentamicin at 0.05ml/kg (5mg/ml) orally and Ca-EDTA 40mg/kg orally after presence of signs (24hrs. post infection).

#### Preparation of antibiotic and Ca-EDTA for invitro activity:

- Preparation of gentamicin:** for invitro activity, 2mg/ml was prepared according to (14).
- Preparation of Ca-EDTA:** For invitro activity, 20mg/ml was prepared according to (15).

**Kirby Baur technique:** At least 4-5 isolated colonies properly isolated and taken from the same morphological type were selected from brain heart infusion agar, those colonies have been transferred to a tube that contain 4-5ml of nutrient broth. The inoculated broth was incubated at 35-37 °C for 18 hrs. until the appearance of visible turbidity. A swab that was sterilized was dipped in the bacteria's standardized suspension, the excessed fluid was expressed through pressing and rotating the swab strongly against the inside of the tube over the level of the fluid. After that, the swab was streaked in 3 directions over the whole surface of the Muller Hinton agar. The inoculated plates were left to dry for five minutes. Then, a multiple antibiotics discs (Amikacin, Augmentin, Gentamicin and trimethoprim) (MASTDISC company, UK), these discs were soaked in Ca-EDTA (20mg/ml) and allowed to dry then transfer on inoculated media.

#### Evaluation of EDTA as an antimicrobial:

Effects of Ca-EDTA (20mg/ml) according to (16) and gentamycin (2mg/ml) both alone and in combination were examined on 24hrs. broth culture of *S. typhimurium*. This test was done in accordance with the recommendations stated by the Clinical and Laboratory Standards Institute (17).

#### Examination of the rabbits:

All rabbits were examined for the following:-

- Daily observation of clinical signs after inoculation of bacteria and after treatment for presence of diarrhea, fever, anorexia and other signs.
- Fecal examination:** Fecal swabs were taken daily from all groups and cultured on media (MaCconkey, nutrient agar, SS agar and brilliant green agar) for evaluation the bacterial shedding.

#### RESULTS

The presented results of invitro activity showed a regular zones of inhibition with using antimicrobials discs (Fig. 1), while it showed a synergistic effect of antimicrobials plus Ca-EDTA with little bacterial growth as (Fig. 2) as compared with using antimicrobials discs alone, and showed a clear zone due to EDTA effect (Fig. 3).

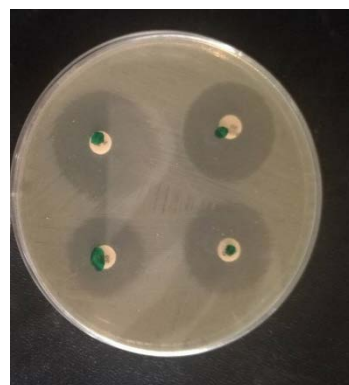


Fig. 1. Antimicrobial discs

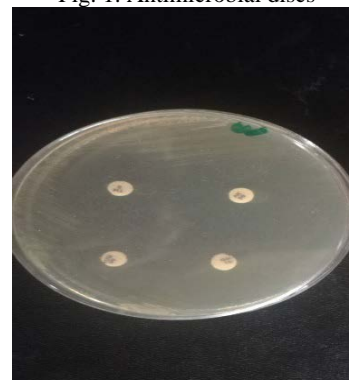


Fig. 2. Discs with Ca-EDTA



Fig. 3. Ca-EDTA alone

Also, The current results showed a clearance of tube containing gentamicin plus Ca-EDTA as compared with control positive tube (tube B.) which indicating an effectiveness of this combination, while in contrary the tubes which containing gentamicin or Ca-EDTA alone, they showed a decrease turbidity as compared with control positive tube (Fig. 4).



Fig. 4. Tubes with bacteria, gentamicin or Ca EDTA alone or combination

After the appearance of clinical signs which mentioned in a previous study (Hasan et al., 2018), the gentamycin treated group showed no response to treatment, also Ca- EDTA alone showed a some improving in health of infected rabbit, while the combination treatment (Gentamicin + Ca-EDTA) showed response to this therapy.

#### DISCUSSION:

The EDTA's antimicrobial effect were explained for a wide domain of clinical microorganisms that contain fungi, amoeba, yeasts, and Gram -positive and -negative bacteria. The integrity regarding the outer leaflet of the outer membrane of the Gram-negative bacteria is handled through hydrophobic lipopolysaccharide (LPS) and LPS-protein interactions. Divalent cations such as  $Mg^{2+}$  are important as they stabilize the negative charges of oligosaccharide chain of the LPS component. It has been proved that EDTA have the ability to remove  $Ca^{2+}$  and  $Mg^{2+}$  ions from the outer cell wall of Gram-negative bacteria, as a result for that, it does liberate up to 50% of the LPS molecules and exposing phospholipids of the inner membrane, increasing the efficiency of other antimicrobials (18).

Many researchers reported that the modes of action regarding EDTAs is to potentiate antibiotics through a disruption process for the lipopolysaccharide structure in outer membrane of the gram-negative bacteria. By this disruption process, the membrane become extra permeable to other agents (9, 10).

Although the results of our study displayed the *in vivo* efficiency of a single dose regimen of gentamicin-EDTA treatment against *Salmonella typhimurium*, moreover, we sought that gentamicin-EDTA would reduce frequency and length of the treatment. These results were in agreement with a result of Chauhan *et al.* (19) who documented the capability of Ca-EDTA chelators to eradicate some bacteria, they experimented Gentamicin-EDTA *in vivo* in rats against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*, also they proved that the use of EDTA (30 mg/ml) as a supplement to the gentamicin (5 mg/ml) antibiotic lock solution were completely eradicated Gram-positive and Gram-negative bacteria.

#### CONCLUSION:

The main conclusion of our data demonstrate that EDTA acts synergistically with gentamicin to treat the animals with salmonellosis by reducing length and frequency.

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