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EFFECT OF ETHYLENE DIAMINE TETRAACETIC ACID (EDTA) ON *IN VITRO* ANTIBACTERIAL ACTIVITY OF TETRACYCLINE AND AMPICILLIN AGAINST *ESCHERICHIA COLI* STRAINS

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SUMMARY

The effect of ethylene diamine tetraacetic acid (EDTA) on *in vitro* antibacterial activity of tetracycline and ampicillin against *E. coli* strains was investigated using the disc diffusion method. Ten multi-resistant *E. coli* strains isolated from chickens were used. Plain and EDTA (1 mg/ml) amended Mueller-Hinton agar plates were inoculated with the test bacteria. The diameter of zones of inhibition around the tetracycline (25 μg) and ampicillin (25 μg) discs on the plain and EDTA Mueller-Hinton media were measured with a metre rule. The significance of the difference in the mean inhibition zone diameter (IZD) was determined using the Student's t-test statistics. The kinetics of inhibition of growth of *E. coli* strain E21 by EDTA-antibacterial combination was determined spectrophotometrically. The absorbance of the culture solution was measured at two-hourly intervals over a period of 10 hours and the values obtained were plotted against time. In the presence of 1 mg/ml of EDTA, tetracycline produced a significantly ($p \leq 0.05$) higher mean IZD in all the test bacteria while ampicillin produced a significantly ($p \leq 0.05$) higher mean IZD in 50% of the ten *E. coli* strains. Profound inhibitory activity of the antibacterial agents in the presence of EDTA occurred during the first four hours of incubation. The results of the study suggest profound synergistic action between tetracycline and EDTA against the test bacteria while the synergistic action between ampicillin and EDTA appears to vary with the strain of *E. coli*. *In vivo* studies on the possible synergistic effect of EDTA and tetracycline against *E. coli* infections are recommended.

KEY WORDS: Tetracycline, Ampicillin, EDTA, Multi-resistant, *E. coli*, Inhibition, absorbance

INTRODUCTION

Resistance to antibacterial agents is a serious setback in the treatment of bacterial infections. Apart from the often expensive option of synthesizing new antibacterial agents against these resistant bacteria, there exist affordable alternative of improving the efficacy of existing agents. Combination of two or more antibacterial agents has proved to be an effective method in combating bacterial resistance (Degener *et al.*, 1986; Klastersky and van den Auwera, 1986). One of the major advantages of using combination therapy is the synergistic activity that is usually produced (Krogstad and Moellering, 1980). Studies on the mechanisms of synergy have shown that drugs which inhibit peptidoglycan synthesis enhance cellular uptake of streptomycin in *E. coli* strains (Plotz and Davis, 1962). Ethylenediamine tetraacetic acid (EDTA) has been known to produce reversal of antibacterial resistance in

resistant strains of Gram-negative bacteria (Brown and Richard, 1965; Weisser *et al.*, 1966; Wooley *et al.*, 1983; Gotthelf, 2003).

In Southeast Nigeria, drug preparations containing ampicillin and tetracycline are widely used in the chemotherapy of bacterial infections (Chah and Nweze, 2001). However, to these agents among *E. coli* strains isolated from chickens has been shown to be high (Blanco *et al.*, 1997; Chah and Oboegbulem, 1998; Al-Ghamdi *et al.*, 1999; Chah *et al.*, 2000; Chah and Okwor, 2003). Thus, combination of each of these agents with EDTA may enhance their antibacterial activity against resistant *E. coli* strains. This study was therefore initiated to evaluate the effect of EDTA on the activity of tetracycline and ampicillin against multi-resistant *E. coli* strains.

MATERIALS AND METHODS

Test bacterial strains

Ten *E. coli* (5 clinical and 5 non-clinical) strains (from exotic chickens) resistant to at least five antibacterial agents including tetracycline and ampicillin were used in this study.

Maximum concentration of ethylenediamine tetraacetate (EDTA) that allowed growth of the test E. coli strains

The maximum concentration of EDTA that supported growth of the test *E. coli* strains was determined by the agar dilution method. A stock solution containing 320 mg/ml of EDTA was prepared by dissolving 3.2g of EDTA powder in 10ml of distilled water. Two-fold serial dilutions of the stock solution were made in distilled water to produce EDTA concentrations ranging from 10 mg/ml to 160mg. Unit volume of each of the EDTA dilutions was added to the 19ml of MH agar to give final EDTA concentrations ranging from 0.5 mg/ml to 8 mg/ml. The agar bottles were appropriately labeled and sterilized by autoclaving at 121°C for 15 minutes. Bottles containing EDTA amended MH agar were allowed to cool to a temperature of 45°C in a water bath and thereafter the agar was poured into sterile Petri dishes, allowed to set and the plates identified appropriately.

Colonies of the test bacteria were suspended in sterile phosphate buffered saline (PBS) and density adjusted to correspond to 0.5McFarland turbidity standard (corresponding to approximately 10^8 cfu/ml). Surfaces of the EDTA-MH agar were spot inoculated with each of the bacterial suspension (i.e. 10 spots per plate). A plain MH agar plate was similarly inoculated, and this served as a negative control. Inoculated plates were incubated at 37°C for 24hrs. After incubation the plates were observed for bacterial growth (i.e. visible colonies). Growth on plain MH agar but not on EDTA amended agar indicated growth inhibition at the particular EDTA concentration. EDTA concentration of 0.5 μg/ml and 1mg/ml allowed growth of all test bacteria. Thus, maximum concentration of EDTA, which permitted growth of all test *E. coli* strain was 1 mg/ml. This was the maximum EDTA concentration used in this study.

Effects of EDTA on the activity of tetracycline and ampicillin against test E. coli **strains**

This was done using the disc diffusion method (Bauer *et al.*, 1966). Plain and EDTA (1 mg/ml) containing Mueller-Hinton agar plates were prepared as described above. Surfaces of the plain and EDTA amended MH agar plates were flooded with the standardized inoculum of the test bacteria. Excess fluid was drained

into a discard pot containing Lysol® disinfectant. Tetracycline (25 μg) and ampicillin (25 μg) discs were applied on the surfaces of inoculated MH agar plates. After incubation at 37°C for 24 hours, inhibition zone diameters (IZDs) around the discs were measured with a metre rule and recorded to the nearest millimetre. Each test was performed in triplicate and the mean 1ZD determined.

Kinetics of inhibition of bacterial growth by EDTA-antibacterial combinations

Escherichia coli strain E21 was used for these kinetics studies. Minimum inhibition concentrations of tetracycline and ampicillin for this strain were 512μg/ml and 1024μg/ml respectively. Three colonies of test strain were inoculated into 10ml of nutrient broth (NB) and incubated at 37°C for 3 hours. Unit volume of the broth culture was added to each of 9 universal bottles containing 19ml of (1) NB (2) NB plus EDTA (1mg/ml) (3) NB plus ampicillin, (4) NB plus tetracycline, (5) NB plus ampicillin plus EDTA (1mg/ml), (6) NB plus tetracycline plus EDTA (1mg/ml), (7) NB plus EDTA (0.5mg/ml), (8) NB plus ampicillin plus EDTA (0.5ug/ml) and (9) NB plus tetracycline plus EDTA (0.5ug/ml).

Inoculated solutions were incubated at 37°C and 2ml samples were withdrawn from each test solution at 0, 2, 6, 8 and 10 hours after incubation and bacterial growth determined spectrophotometrically (Spectrosonic 21D, Milton Roy, USA) by measuring the absorbance of the samples at 630nm (Wooley *et al.*, 1981). Each test was conducted in triplicate and the mean absorbance at each time period calculated.

Growth curves of *E. coli* E21 in the presence and absence of EDTA and antibacterial agents were produced by plotting mean absorbance at 630nm wavelength against length of incubation.

Statistical analysis

For each strain the significance of the difference in mean 1ZD produced by tetracycline and ampicillin in the presence and absence of 1 mg/ml EDTA was determined using the Student t-test statistics (GraphPAD Software). Significant differences were considered at 5% probability level.

RESULTS

Effect of EDTA on antibacterial activity of tetracycline and ampicillin

The mean inhibition zone diameters produced by ampicillin and tetracycline alone and in combination with EDTA (1 mg/ml) against the test *E. coli* strains are shown in Tables I and II. In the presence of the 1 mg/ml of EDTA, tetracycline (25 μg) caused significantly ($p<0.05$) higher mean 1ZD in all the bacterial strains tested. However, incorporation of EDTA at a concentration of 1 mg/ml to MH agar resulted in a significant ($p<0.05$) increase in the mean 1ZD produced by ampicillin (25 μg) in 5 (50%) of the 10 *E. coli* strains.

TABLE I: Effect of EDTA (1mg/ml) on the inhibition zone diameter (IZD) produced by tetracycline disc (25µg) against multi-resistant avian *E. coli* strains

E. coli strain	Mean IZD (±SEM)		t-test value
	Plain agar	*EDTA agar*	
E20	9.33 ±0.33	15.33±0.33	12.73***
E21	7.67 ±0.33	20.33 ±0.33	26.87***
E26	9.00 ±0.58	28.67 ±0.67	14.76***
E40	8.67 ±0.67	10.33 ±0.33	2.24*
E41	9.67 ±0.89	18.33 ±0.88	6.95**
20	9.67 ±0.33	31.00 ±0.58	32.00***
28	7.67 ±0.68	18.67 ±0.67	11.67***
31	7.33 ±0.33	20.33 ±0.88	13.79***
285	10.3 ±0.33	12.33 ±0.33	2.68*
323	8.33 ±0.33	24.00 ±0.58	23.50***

SEM = Standard error of the mean; *** p<0.001; ** p<0.01; * p<0.05

TABLE II: Effect of EDTA (1mg/ml) on the inhibition zone diameter (IZD) produced by ampicillin disc (25µg) against multi-resistant avian *E. coli* strains

E. coli strain	Mean IZD (±SEM)		t-test value
	Plain agar	*EDTA agar*	
E20	8.33 ±0.33	8.67 ±0.67	0.45^{ns}
E21	8.00 ±0.58	14.67 ±0.67	7.56***
E26	7.68 ±0.33	9.00 ±0.58	2.00^{ns}
E40	7.68 ±0.67	9.67 ±0.67	2.12*
E41	7.68 ±0.33	10.33 ±0.33	5.66***
20	9.33 ±0.33	6.33 ±0.67	0.45^{ns}
28	8.33 ±0.67	8.67 ±0.33	0.45^{ns}
31	8.33 ±0.33	12.33 ±0.33	8.49***
285	12.33 ±0.33	12.67 ±0.67	0.45^{ns}
323	9.00 ±0.58	10.67 ±0.33	2.50*

SEM = Standard error of the mean; *** p<0.001; ** p<0.01;
*** p<0.05;**
^{ns} Not significant (p>0.05)

Kinetics of growth inhibition

The kinetics of growth inhibition of *E. coli* strain E21 by tetracycline and ampicillin alone and in combination with 1mg/ml of EDTA is shown in Fig. 1. During the first four hours of incubation, minimal increase in absorbance was observed for culture solutions containing tetracycline-EDTA and ampicillin-EDTA combinations compared with the respective absorbance at start of the experiment. Such observations were not obtained with solution containing the test antibacterial agents alone or EDTA alone. However, after 4 hours of incubation, a steady increase in the absorbance of tetracycline-EDTA or ampicillin-EDTA solutions was observed. At the end of the experiment (after 10 hours of incubation) the absorbance for tetracycline-EDTA solution was 0.166, while that of ampicillin-EDTA was 0.209. The values for EDTA alone,

tetracycline alone, ampicillin alone and plain broth were 0.236, 0.300, 0.310, and 0.354 respectively.

The kinetics of inhibition of this bacterial strain by the test antibacterial agents alone and in combination with 0.5mg/ml of EDTA is presented in Fig. 2. As shown in this figure, only tetracycline-EDTA produced growth curve similar to the curve observed in Fig. 1. However, after the observation period the absorbance of the tetracycline-EDTA (0.5mg/ml) was 0.235 as against the 0.166 recorded for tetracycline/EDTA (1mg/ml).

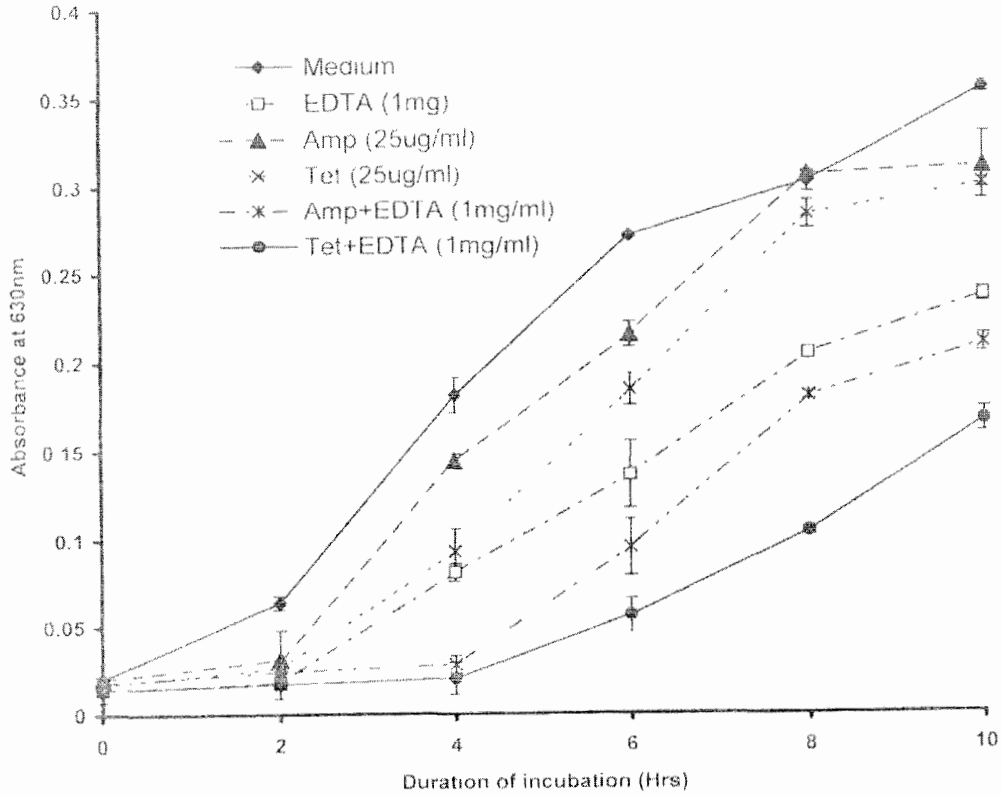

Fig. 1: Growth curves of *E. coli* strain E21 in the presence of test antibacterial agents and EDTA (1mg/ml)

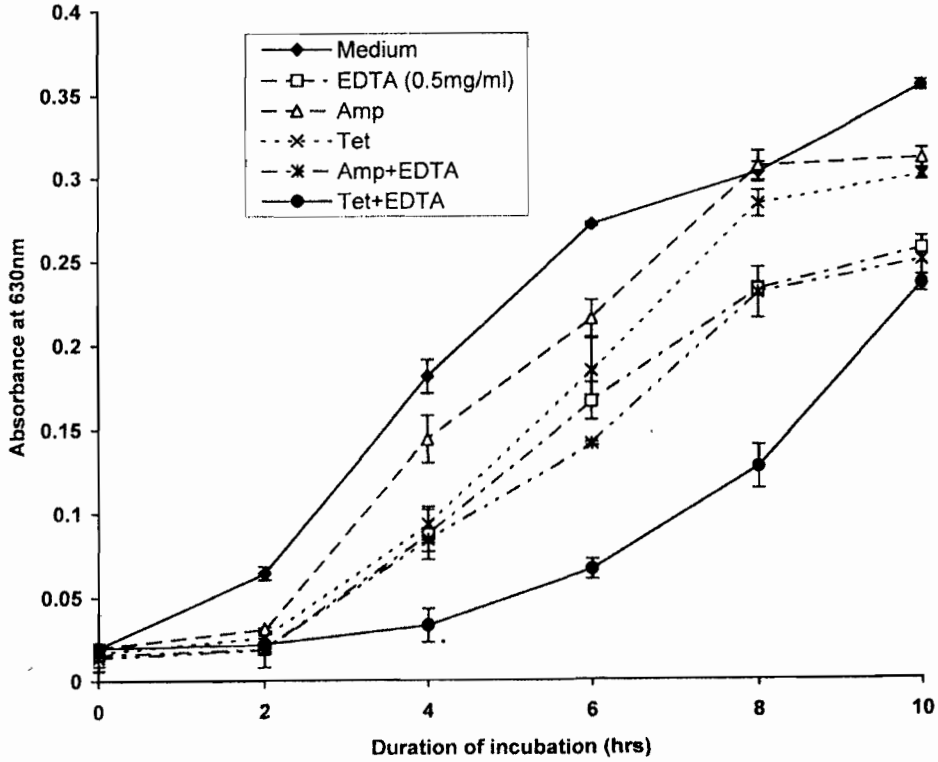

Fig. 2: Growth curves of *E. coli* strain E21 in the presence of test antibacterial agents and 0.5mg/ml of EDTA

DISCUSSION

Exposure of Gram-negative bacteria to EDTA results in damage of cell surfaces (Roberts *et al.*, 1970) and treatment of *E. coli* with this agent may result in a release of 50% of the envelope lipopolysaccharide (Bayer and Leive, 1977). Alteration in the cellular architecture of Gram-negative bacteria following exposure to EDTA increases permeability of the bacteria to extracellular solutes and leakage of intracellular solutes (Leive, 1968). Also, exposure of these organisms to EDTA–tromethamine results in increased sensitization of the bacteria to antibiotics (Brown and Richard, 1965, Wheat, 1984). Ampicillin interferes with bacterial cell wall formation by preventing the cross linking of peptidoglycans while EDTA interferes with biosynthesis of peptidoglycans by chelating Mg^{2+} (Lambert, 1992). Thus, the site of activity of these agents may explain their synergistic action on Gram-negative bacteria. Increased inhibition of *E. coli* by EDTA–ampicillin combination may also be due to the fact that damage of cell surface resulted in increased uptake of ampicillin by the bacterial cell. Damage of cell surface also resulted in loss of periplasmic beta-lactamase enzymes thus leading to accumulation of ampicillin in

the periplamic space with its attendant interference with cell wall synthesis. Since reduce permeability is a common mechanism of resistance to the tetracyclines in Gram-negative bacteria (Nikaido, 1989; Webber and Piddock, 2001), the profound inhibitory action of tetracycline in the presence of EDTA possibly resulted from increased penetration of tetracycline or loss of drug inactivating enzymes following EDTA effect on permeability barrier.

The synergistic activity of EDTA and the test antibacterial agents was further confirmed spectrophotometrically in growth inhibition kinetic studies. Growth of the test bacterium in the solution resulted in increased turbidity, which was indicated by increase in the absorbance. As shown in Figs. 1 and 2 changes in the absorbance for tetracycline–EDTA) and ampicillin–EDTA growth solutions were minimal during the first 4 hours of incubation, thus indicating minimal growth during this period. However, after 4 hours incubation, the steady increase in the absorbance recorded suggests that some cells in the bacterial population were capable of overcoming the inhibitory activity of EDTA–antibacterial combinations and thus were able to grow and multiply. Nonetheless, bacterial growth in these three solutions was lower than in the plain solutions or media containing the antibacterial agents or EDTA alone. Ethylendiamine tetraacetic acid (1mg/ml and 0.5mg/ml) on its own produced higher inhibitory action on bacterial growth than the respective antibacterial agents. This observation is not surprising since *E. coli* have been reported to be relatively sensitive to the effects of EDTA (Wooley and Blue, 1975).

Combination of oxytetracycline and EDTA–tromethamine has been demonstrated to have profound inhibitory effects on *E. coli* and *P. aeruginosa* growth (Wooley *et al.*, 1981; Wooley and Jones, 1983). Wooley *et al.* (1981) and Wooley and Jones (1983) recorded significant inhibitory actions of oxytetracycline and EDTA–tromethamine combination throughout their ten hours' observation period. However, in the present study such profound effects were observed only during the first four hours of incubation. This difference may be attributed to the fact that the earlier authors included tromethamine in the drug combination. Tromethamine has been reported to enhance the effect of EDTA (Goldschmidt and Wyss, 1967).

Colibacillosis is increasingly becoming a serious problem in the poultry industry worldwide. Resistance of *E. coli* strains to readily available and affordable antibacterials is also a major threat to effective treatment of these infections. The need to overcome the threat posed by these emerging resistant bacterial infections cannot be overemphasized. The results of the present study as well as those of Wooley *et al.* (1981) and Wooley and Jones (1983) have demonstrated that EDTA significantly potentiates the activity of tetracycline and ampicillin against resistant *E. coli* strains. Thus, one of the possible options of surmounting the antibacterial resistance problem will be to combine available and affordable antibacterials with potentiating agents such as EDTA. Such combinations have been effectively used in the treatment of coliform cystitis in humans (Goldschmidt *et al.*, 1972), otitis externa (Blue *et al.*, 1974), bacterial rhinitis in dogs (Wooley *et al.*, 1979) and multiple fistulas in dogs (Bjorling and Wooley, 1982). Farca *et al.* (1997) reported on successful application

of EDTA-tromethamine and cephaloridine, kanendomycin and enrofloxacin in the treatment of cases of chronic otitis externa, chronic dermatitis and recurrent cystitis in dogs.

The results of this study have shown that of ETDA significantly potentiated the activity of tetracycline and to some extent ampicllin against multi-resistant avian *E. coli* strains. Combination of tetracycline or ampicillin with EDTA may be exploited in the treatment of cases of colibacillosis in poultry. However, *in vivo* studies on the effects of EDTA/antibacterial combination on experimental and natural avian *E. coli* infections are recommended.

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