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Diclofenac in the Management of *E. coli* Urinary Tract Infections

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Abstract. *E. coli is the main agent of uncomplicated urinary tract infections (UTIs) and accounts for more than 85% of recurrent cystitis and at least 35% of recurrent pyelonephritis. Despite the widespread availability of antibiotics, UTIs remain the most common bacterial infection in the human population. It is currently advised that the clinical administration of antibiotics against the pathogenic bacteria should be prohibitted due to the emergence of multidrug resistant (MDR) bacterial strains. Therefore, newer and more effective antimicrobials are in demand to treat such cases. One hundred and thirty six urine samples were collected from UTI patients. E. coli was isolated from 85 samples, out of which 33% were resistant to common antibiotics. The isolates were decreasingly resistant to ampicillin, tobramycin, augmentin, nalidixic acid, cefuroxime, nitrofurantoin, kanamycin, pipemidic acid, chloramphenicol, cefotaxime, cefamendol, ofloxacin, ceftizoxime, norfloxacin and amikacin. The anti-inflammatory drug diclofenac exhibited significant antibacterial activity against common bacterial strains both in vitro and in vivo. The present work was conducted to evaluate the in vitro inhibitory effect of this drug on the clinically isolated strains of E. coli in hospitals. All the isolates were sensitive to diclofenac, with MIC values ranging from 5-50 μg/mL. The MIC90 value of the drug was 25 μg/mL. Therefore, it may be suggested that diclofenac has the capacity to treat UTI caused by E. coli.*

Escherichia coli has been recognized as an important potential pathogen in humans (1). *E. coli* forms part of the normal microbial flora of the intestinal tract of humans and animals, yet it can be found in water, soil and vegetation. It is not normally pathogenic but may be referred to as an opportunistic pathogen often associated with urinary tract infections (including cystis, pyelitis and pyelonepheritis). *E. coli* is the main causative agent of uncomplicated urinary tract infections (UTIs) and accounts for more than 85% of recurrent cystitis and at least 35% of recurrent pyelonephritis (2). The reservoir for uropathogenic *E. coli* is fecal flora, from which the bacteria spread to the urogenital mucosa, ascend into the bladder and adhere to it. The bacteria multiply and develop a local infection (cystitis) and may further ascend to involve the ulcers and kidneys (pyelonephritis) (3). UTI is the most common bacterial infection in women and occurs with much greater frequency among elderly women with increasing frequency among postmenopausal women (4).

Despite the widespread availability of antibiotics, UTIs remain the most common bacterial infection in the human population. It currently advised that the clinical administration of antibiotics, such as nitrofurantoin, ceftizoxime, ofloxacin, pipemidic acid, nitrofurantoin, cefamendol, cefotaxime, chloramphenicol, kanamycin, cefuroxime, nalidixic acid, augmentin, tobramycin and ampicillin, against the pathogenic bacteria be gradually prohibited due to emergence of multidrug resistant (MDR) bacterial strains (5, 6).

The pharmaceutical industry historically capitalized on the discovery that many microbial secondary metabolites act as antibiotics (7-9). However, massive and often irrational use of antibiotics and antibacterial chemotherapeutics for extended periods has led to the emergence of drug-resistant microorganisms. Scientists have realized that there is an urgent need to search for antimicrobial properties in compounds other than antibiotics, in addition to discovering newer and more powerful antibiotics.

Studies directed along this line revealed that a variety of compounds, employed in the management of pathological conditions of a non-infectious etiology, exhibit broad-spectrum antimicrobial activity *in vitro,* as well as *in vivo* against a variety of Gram-positive and Gram-negative bacteria. Such compounds have been called the "Non-antibiotics" (10).

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The organized investigation of non-antibiotics has shown that antihistamines, such as bromodiphenhydramine and diphenhydramine (11), methdilazine (12), promethazine (13) and trimeprazine (14), tranquilizers like promazine (15), antihypertensives like methyl-DOPA (16), dobutamine (17), amlodipine (18), oxyfedrine (19) and propranolol (20), antispasmodics like dicyclomine (21, 22), antipsychotics like chlorpromazine (23), fluphenazine (24) and thioridazine (25) and the anti-inflammatory agent diclofenac (Dc) (26) possess moderate to powerful antibacterial activity both *in vitro* and *in vivo*. It was reported that aspirin could inhibit the growth of *Klebsiella pneumoniae* at concentrations within the range of that in plasma in normal clinical usage (27). Time-killing studies of *Helicobacter pylori* were performed with different concentrations of aspirin and salicylate and the effects of aspirin on the efficiency of colony formation and metronidazole-induced mutation to rifampicin-resistance were studied. Aspirin inhibited the growth of *H. pylori*, suppressed the mutagenic effect of metronidazole and enhanced the susceptibility of *H. pylori* to antimicrobial agents (28).

The anti-inflammatory agent Dc (Figure 1) exhibited noteworthy inhibitory action against both drug-sensitive and drug-resistant clinical isolates of several Gram-positive and Gram-negative bacteria. This drug was tested *in vitro* against 397 bacteria, most of which were inhibited by the drug at 25-100 μg/mL concentrations. When tested *in vivo*, Dc could significantly protect mice (weighing 20 g each) challenged with a 50 median lethal dose of *Salmonella typhimurium* NCTC 74, when injected at doses of 1.5 and 3.0 μg/g body weight of the animals. The *in vivo* data were highly significant according to the χ^2 test ($p<0.01$). The drug was bactericidal in action, both against Gram-positive, as well as Gram-negative bacterial strains (26). The antibacterial action of diclofenac was found to be due to the inhibition of DNA synthesis, which was demonstrated using 2 μ Ci (^{3}H) deoxythymidine uptake (29). Furthermore, Dc exhibited *in vitro* synergism with the conventional antibiotic streptomycin and with the non-antibiotic antipsychotic drug trifluoperazine against *S. aureus* NCTC 6571 and *E. coli* K12 C600. When compared with their individual effects, the synergism was found to be statistically significant ($p<0.001$). By the checkerboard assessment procedure, the fractional inhibitory concentration index of these combinations was found to be 0.49 and 0.37, respectively, confirming synergism. Both the drug-combinations were significantly synergistic *in vivo* as well (30, 31). The present study was designed to examine whether the drug-resistant clinical strains of *E. coli*, isolated from the UTI cases, were susceptible to Dc.

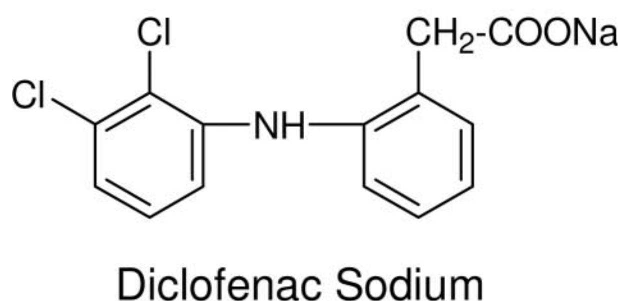

Figure 1. *Structure of diclofenac (Dc).*

Materials and Methods

Isolation of bacterial strains. One hundred and thirty six urine samples were collected from UTI patients in different hospitals in Calcutta, India. All the samples were screened for presence of *E. coli*. The samples were streaked onto MacConkey agar and incubated at 37˚C overnight. Characteristic colonies were identified based on the ability of *E. coli* to ferment lactose, giving rise to pinkish colonies. Special verification was done by biochemical (indole, citrate, urease, triple sugar iron) tests and serological typing (32). *E. coli* K12C600, *E. coli* V517 and *E. coli* ATCC 259222 (used as the sensitive control) were obtained from Central Drug Laboratory of Calcutta, India.

Drugs. All the drugs were obtained as pure dry powder from their respective manufacturers in India and stored at 4˚C. The antibiotic discs were obtained from HI Media, India.

Media. The liquid media used in the study were peptone water [PW; 1.0% bacteriological peptone (Oxoid) plus 0.5% NaCl (Analar)], nutrient broth (NB, Oxoid) and Mueller-Hinton broth (MHB, Oxoid). The solid media were peptone agar (PA), nutrient agar (NA) and Mueller-Hinton agar (MHA), which were prepared by solidifying PW, NB and MHB, respectively with the help of 1.5% agar (Oxoid No. 3).

Inoculum. All the bacterial strains were grown overnight (24 hours) in PA / NA / MHA at 37˚C and were harvested during the stationary growth phase. From these cultures, the organisms were directly suspended in 5 mL sterile distilled water. The turbidity of each suspension was adjusted to match with a 0.5 McFarland standard (33) with the help of a spectrophotometer (Chemito UV 2600 Double Beam UV-Vis Spectrophotometer) at 625 nm, which corresponded to 2.4×10^8 cfu (colony forming units)/mL. The suspension was further diluted 1:100 with sterile distilled water.

Determination of antibiotic susceptibility pattern of the isolates. Antibiotic sensitivity testing was performed by Kirby Bauer technique on MHA plate and the interpretation of results was performed according to NCCLS guidelines, 2003 (34).

Determination of Minimum Inhibitory Concentration (MIC) of Dc. The MIC of Dc against the clinical isolates was accurately determined by the broth dilution method (35). For this detection, 0.1 mL of a standardized suspension of each strain [10^6 colony forming units (cfu/mL)] was added to a tube containing Dc at concentrations of 0 (control), 2, 5, 10, 25, 50 and 100 μg/mL in MHB. These were incubated at 37˚C for 24 hours and examined for visible growth after gentle vortexing of the tubes. The lowest concentration of Dc in a tube that failed to show visible growth

was considered as its MIC. The MIC determination was performed in triplicate for each strain and the experiment was repeated when necessary.

Determination of the mode of action of diclofenac on Escherichia coli ATCC 259222. *E. coli* ATCC 259222 was grown in NB overnight at 37AC. From this culture, 2 mL was added to 4 mL of fresh NB and incubated for 2 hours so that the culture could attain the logarithmic growth phase. The number of viable cells (cfu) was then determined and Dc was added at a concentration higher than its MIC value with respect to *E. coli* ATCC 259222. The cfu counts were determined upto 6 hours at intervals of 2 hours and then after 18 hours (36).

Measurement of macromolecular synthesis in the presence or absence of Dc. The antibacterial agents, whether bacteriostatic or bactericidal, might act by: (i) inhibition of microbial cell wall synthesis, (ii) alteration of membrane function or membrane damage, (iii) Inhibition of nucleic acid synthesis, (iv) Inhibition of protein synthesis.

E. coli K12C600 was grown at 37˚C in nutrient broth; after 18 hours incubation, 1 mL of culture was mixed with 6 mL of fresh broth containing 2 μ Ci (3H) deoxythymidine (specific gravity of 13.5 Ci / m mole). The mixture was shaken at 37˚C to accelerate growth. After 2 hours, 1 mL aliquot was removed, mixed with 100 μL of TCA and kept on ice for determining the initial counts. To the remaining 6 mL broth culture, Dc was added at 2xMIC of the test strain and the mixture was incubated with shaking at 37˚C. At intervals of 30 min, a 1 mL sample was removed, mixed with 100 μL of TCA and was kept on ice. After 5 hours, all the deposits were washed twice individually with 5 mL of 10% TCA and filtered through a Millipore filtration system. The filter pads were then dried at 37˚C and radioactivity was measured in a scintillation counter. A broth culture treated with 2 μ Ci (^{3}H) deoxythymidine, containing no Dc, was used as control.

Animal experiments. Systemic infections were produced in groups of 20 inbred Swiss Albino male mice (*ca*. 18–20 g), abiding by ethical guidelines. The animals were maintained in animal house at standard conditions at 21±1˚C and 50-60% relative humidity with a photoperiod of 14 hours and then 10 hours of light-darkness. Water and a dry pellet diet were given *ad libitum*. The test bacterial strain was *Salmonella typhimurium* NCTC 74, which is naturally virulent to mice. The virulence of the strain was enhanced after repeated passage through mice. The median lethal dose (MLD/LD_{50}) of the passaged strain was determined by injecting graded challenges in batches of mice and recording the mortality upto 100 hours. Freeze-drying and reconstitution did not affect the MLD. The 50MLD of the passaged strain, corresponding to $0.95x10^9$ cfu/mouse, suspended in 0.5 mL NB, served as the challenge dose (37). Standardizing its optical density at 640 nm in a Klett-Summerson colorimeter ensured reproducibility of the challenge dose.

Determination of toxicity of Dc. Sixty mice were taken, 20 of which were injected 15 μg of Dc per mouse, 20 received 30 μg of the drug per mouse and the remaining 20 were given 60 μg of the drug per mouse. They were kept under observation up to 100 hours.

Determination of protective capacity of Dc. Sixty mice were divided into 3 groups, of 20 animals per group. Each animal in Group I was injected 15 μg of Dc, Group II received 30 μg and Group III was given 60 μg of the drug per mouse. Three hours after injection of the drug, each animal was challenged with 50MLD of *S. typhimurium* NCTC 74. A control group of 60 animals was also simultaneously administered with *S. typhimurium* and 0.1 mL sterile saline in place of Dc. The protective effect of the drug was assessed by recording the mortality of animals in different groups within 100 hours of treatment.

In another experiment, 4 groups of mice, each consisting of 6 animals were taken. All the mice received the challenge dose of bacteria; Groups 1 and 3 were given 15 μg of Dc 3 hours prior to the challenge, while Groups 2 and 4 were injected sterile saline. Groups 1 and 2 were sacrificed 2 hours after the challenge, their livers and spleens were removed aseptically, homogenized in sterile glass homogenizers and then the cfu count was determined from each sample individually. The same procedures were also followed for Groups 3 and 4 after 18 hours. The statistical analysis of the data was done according to the method of Bhattacharya and Johnson (38).

Isolation of plasmid DNA. For physical demonstration of plasmid DNA in wild-type strains as well as step-up mutant strains, the extracted plasmid DNA samples were electrophoresed (39).

Production of step-up mutants. The bacterial strains were allowed to grow in media containing concentrations of Dc higher than its MIC towards that strain. Thus, by using successively higher concentrations of Dc, step-up mutants of that strain with respect to Dc were obtained. All the mutants were tested for their sensitivity or resistance to the conventional antibiotics, such as ampicillin (Ap), tobramycin (Tm), augmentin (Au), nalidixic acid (Na) and chloramphenicol (Cm).

Results

Antibiogram pattern of the isolates. Strains of *E. coli* were normally sensitive to most of the antibiotics and chemotherapeutics. About 55% isolates were susceptible to most of the antibacterial drugs, while 15% showed intermediate susceptibility; 33% of the isolates were found to be resistant. The antibiogramme resistance pattern of the strains, as given in Table I, was: ampicillin (74.4%), tobramycin (62.5%), augmentin (59%), nalidixic acid (48%), chloramphenicol (42%), cefotaxime (38%), cefamendol (32.5%), cefuroxime (31%), nitrofurantoin (26%), kanamycin (25%), pipemidic acid (24%), ofloxacin (15.4%), ceftizoxime (12.5%), norfloxacin (7.9%) and amikacin (0). These findings were in accordance with the results of Samsygina *et al.* and Khan (40, 41).

In vitro antimicrobial action of Dc. Dc was tested against a total of 85 isolates of *E. coli* (Table II), out of which, 7 were inhibited at 2 μg/mL of Dc, 12 at 5 μg/mL, 20 strains at 10 μg/mL, 9 strains each at 25 and 50 μg/mL and the remaining 28 strains of *E. coli* were resistant to Dc.

In vivo experiments with Dc. As shown in Table III, the control group (60 mice), 48 animals died within 100 hours of injecting the challenge dose. In the Dc-treated groups,

Table I. *Antibiogram sensitivity pattern in E. coli.*

Drug	Sensitivity (%)
Ampicillin	74.4
Tobramycin	62.5
Augmentin	59
Nalidixic acid	48
Chloramphenicol	42
Cefotaxime	38
Cefamendol	32.5
Cefuroxime	31
Nitrofurantoin	26
Kanamycin	25
Pipemidic acid	24
Ofloxacin	15.4
Ceftizoxime	12.5
Norfloxacin	7.9
Amikacin	0

Table II. *Inhibitory spectrum of diclofenac (Dc).*

Bacteria	No. tested	No. of strains inhibited by Dc at (μg/mL)					
		2	5	10	25	50	100
E. coli	85	7	12	20	9	9	28

there was a significant protection, according to the Chi-square test ($p<0.001$ for 60 μg and 30 μg dose, and $p<0.01$ for 15 μg of Dc). The mortality rate was very low in those groups of mice administered different doses of Dc only.

The results presented in Table IV clearly indicate that treatment with Dc significantly reduced the cfu/ml of bacteria in the organs of the mice, both at 2 hs and 18 hs after the challenge dose, compared with the saline-treated group (control). On the basis of statistical analysis, it was found that $p<0.005$ for the 2-hs samples and $p<0.01$ for the 18 hs samples.

Kinetic studies on the action of Dc. The MIC of Dc against *E. coli* ATCC 259222 was found to be 10 μg/mL. At the logarithmic growth phase of the culture, when the cfu count of the strain was $4.6x10^8$, 20 μg/mL of Dc were added to the culture. Bactericidal action was noted, as the cfu count was $9.0x10^7$ after 2 hs, 1.0x107 after 4 hours, $1.0x10^5$ after 6 hours and 0 at the end of 18 hours (Figure 2).

Determination of radioactive count in the bacterial culture. The break point of cellular DNA after incorporation of Dc was measured by the loss of TCA-perceptible radioactivity. At 30-min intervals, after the addition of Dc, the TCA-perceptible radioactivity was found to exhibit a gradual decline in the counts/min (Figure 3). No degradation of cellular DNA was observed when *E. coli* K12C600 cells were not treated with Dc.

Dc-resistant mutants of bacterial spp. and their effect on changing the resistance pattern to antibiotics. It was observed that there was a noticeable reduction in the MIC values of the antibiotics with respect to the same strains, compared to their MIC values previously determined (Table V). Agarose gel electrophoresis of plasmid DNA extracted from donor (wild-type) and recipient (step-up mutant) clinical isolates of *E. coli* along with reference *E. coli* V517 showed the absence of any specific plasmid band in the step-up mutant (Figure 4).

Discussion

The findings of this study revealed that *E. coli* strains collected from UTI patients were normally sensitive to most of the antibiotics and chemotherapeutic agents. About 55% of the isolates were susceptible to most of the drugs, while 15% showed intermediate susceptibility and only 33% were found resistant. These results are in conformity with those of Hameed *et al.*, and Sotto (42, 43). However, these findings showed that none of the drugs is effective against all the isolates of *E. coli*. For this purpose, susceptibility tests should be carried out by clinicians, based on the sample, to ensure the prescription and use of the most effective antibiotic.

The phenylacetic acid derivative diclofenac displays analgesic, antipyretic, as well as anti-inflammatory properties. This non-steroidal anti-inflammatory drug has demonstrated strong antimicrobial property when tested against a large number of Gram-positive and Gram-negative bacteria, the MIC ranging from 50-200 μg/mL in most of the cases and even lower in some instances (26). Dc is bactericidal in nature (26, 29). The drug could also offer significant protection to mice challenged with a virulent bacterium. Although it is reported to be a somewhat toxic agent for human consumption, the drug was well-tolerated by mice (26, 30, 31). Protection of the animals at low concentrations of the drug could be achieved probably due to the fact that Dc is rapidly and completely absorbed after oral administration. There is a considerable first pass effect, such that only about 50% of the drug is available systematically. Its half-life in plasma is 1 to 2 hours. Dc produces side-effects in only 20% of patients when used as an anti-inflammatory agent and only 2% of them discontinue therapy as a result (44). These effects depend on genetic and nutritional factors, as well as the physiological state of the patient.

The antibacterial activity of Dc is due to its inhibition of bacterial DNA synthesis, which was demonstrated using 2 μ Ci

Table III. *Mouse-protective capacity of diclofenac (Dc).*

Control Group*		Test Groups*		Control Groups**	
Without Dc	No. deaths (60)	Dc (μg) injected / mouse	No. deaths (20)	Dc (μg) injected / mouse	No. deaths (20)
0.1 mL saline	48	60	5[a]	60	3
		30	6[a]	30	4
		15	7[b]	15	1

* Received a challenge dose of $0.95x10^9$ cfu in 0.5 mL NB of *S. typhimurium* NCTC 74;
** Received no challenge.
[a]$p<0.001$.
[b]$p<0.01$, according to Chi-square test.
The mortality rate was very low in those groups of mice that were administered different doses of Dc only.

Table IV. *Reduction in cfu/mL of S. typhimurium NCTC 74 in organ homogenates of mice treated with diclofenac (Dc).*

Time of sampling	Group	Mouse No.	Drug (μg/mouse)	Cfu/mL counts in	
				Liver	Spleen
2 hours	1	6	Dc (15 μg)	$1.2x10^2$-$2.6x10^3$	$2.6x10^2$-$4.6x10^3$
	2	6	Saline (0.1 mL)	$2.6x10^4$-$6.9x10^5$	$2.5x10^4$-$4.2x10^5$
18 hours	3	6	Dc (15 μg)	$3.8x10^3$-$9.6x10^4$	$1.2x10^3$-$7.2x10^5$
	4	6	Saline (0.1 mL)	$3.9x10^6$-$9.6x10^7$	$2.4x10^6$-$9.2x10^7$

Data analyzed statistically (Student's '*t*' test) $p<0.005$ for 2- samples and $p<0.01$ for 18 hours samples.

Table V. *Reversal of resistance in diclofenac (Dc) mutants.*

E. coli V517	Dc wild-type	Dc	Tm	Au	Na	Cm	Ap
		25	8	8	4	8	8
	Dc step-up 1 (100)	50	50	8	2	8	8
	Dc step-up 2 (200)	150	50	4	2	8	8
	Dc step-up 3 (400)	500	100	4	2	2	8

Tobramycin (Tm), augmentin (Au), nalidixic acid (Na), chloramphenicol (Cm), ampicillin (Ap).
Figures in parenthesis indicate selective concentrations of Dc for deriving Dc mutants of corresponding levels of resistances. The step 2 mutants were derived from corresponding step 1 mutants.

(^{3}H) deoxythymidine uptake (29). The synergism between diclofenac and streptomycin against *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* K12 C600 was found to be statistically significant ($p<0.01$), when compared with their individual effects. By the checkerboard assessment procedure, the FIC index of this combination against *Escherichia coli* K12 C600 was found to be 0.49, confirming synergism (30). The degree of synergism between diclofenac and the phenothiazine non-antibiotic trifluoperazine provided statistically significant ($p<0.001$) values; FIC value was 0.37 against *Escherichia coli* K12 C600 (31). The mouse protective capacity of both these combinations suggested them to be highly synergistic (30, 31). There was a noticeable reduction in the MIC values of the antibiotics with respect to those bacterial strains treated with Dc, when compared to their MIC values previously determined against the same untreated strains. This demonstrates that diclofenac eliminates natural resistance in common bacterial pathogens to specific antibiotics.

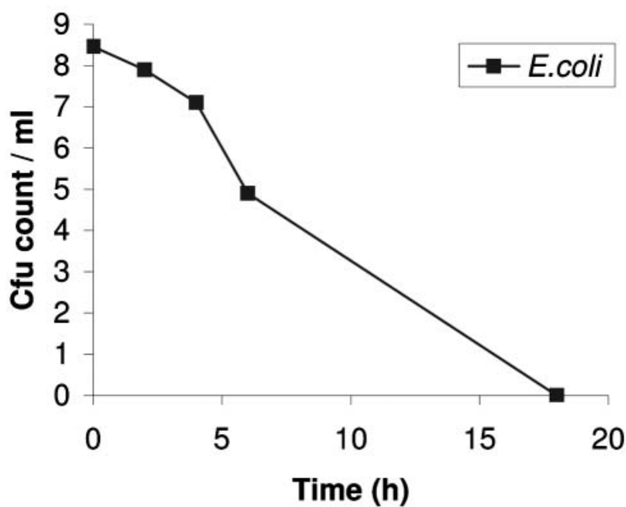

Figure 2. *Bactericidal action of diclofenac (20 mg/mL) on E. coli ATCC 259222.*

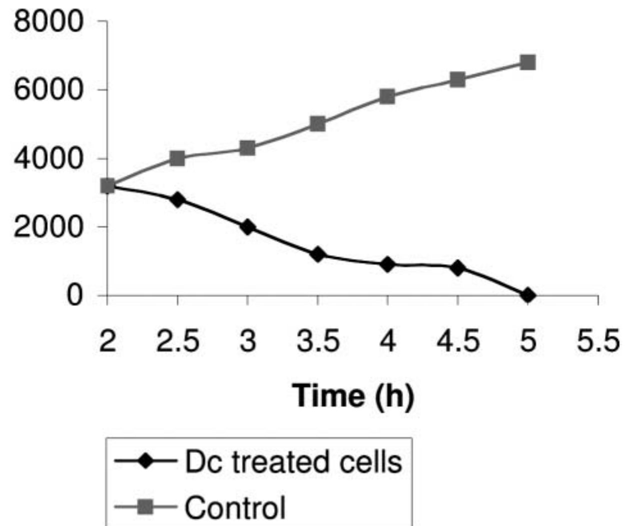

Figure 3. *Effect of diclofenac (Dc) on DNA synthesis using E. coli K12C600.*

Therefore, diclofenac is suggested to possess the capacity to treat urinary tract infections caused by drug-resistant *E. coli* strains. Furthermore, in time, it may be possible to combine this drug synergistically with prospective antibiotics or other compounds, thereby developing a new class of potential weapons against UTI.

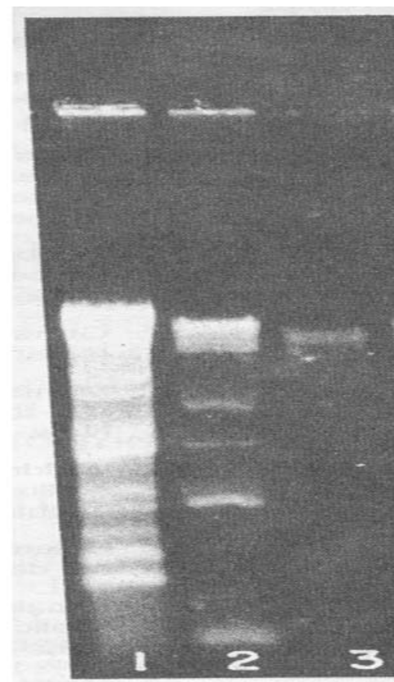

Figure 4. *Agarose gel electrophoresis of plasmid DNA. Lane 1 shows 7 distinct plasmid bands of E. coli V517. Lane 2 shows several plasmid bands in a wild-type strain of E. coli, Lane 3 shows the absence of any specific plasmid band in the step-up mutant of the same clinical isolate of E. coli.*

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