Antimicrobial Resistance studyon*E. coli* Isolated from Cattle Egrets Foragingin Livestock Farm of Ayodhya District

Abstract

This study was undertaken to establish the role of cattle egretforaging nearby animal farm as a possible source of MDR bacteria. A total of 50 faecal dropping samplesof cattle egret were collected from cattle farm and processed in the laboratory for isolation of *E. coli*. Total45 isolates were recovered,of which 20 were identified and characterized as *E. coli*morphologically and biochemically.Antibiogram profile of all *E. coli*isolates recovered from cattle egretrevealed100% resistancefor Ampicillin, Cloxacillin and Cefotaxime. Antibiotics like Imipenem, Gentamycin, Trimethoprim and Triple sulphas were found highly sensitive (100%) followed by Streptomycin (90%). Sensitivity of Norfloxacinand Kanamycin ranged between intermediate to high degree,whereas Polymyxin was found sensitive for 55% isolates. Multi antimicrobial resistance index of all the isolates was found to be more than 0.27.These, MDR positive *E. coli* were further subjected to phenotypic and genotypic confirmation of ESBL producersby DDST and PCR, respectively.Out of 20,12 isolates (60%) were confirmed as ESBL producers by DDST and PCR. The PCR analysis of ESBL positive isolates revealed predominance of bla_{CTX} gene. All *E. coli* were also assessed for pathogenicity by in vitro tests and 50% and 40% isolates were found to be pathogenic in Congo red binding and haemolysis test, respectively. In the present study, occurrence of MDR *E. coli* and ESBL producers in cattle egret is a cause of concern for public health and animal health.

Key Words: *E. coli,* Antimicrobial Resistance, ESBL, Cattle Egret, Pathogenicity test

1. Introduction

Antimicrobial resistance (AMR) is one of the top ten threats to public health, as listed by the World Health Organization (**1**). Today, antimicrobial resistance has been a major challenge for pharmaceuticals, clinicians and researchers due to their indiscriminate and extensive use in human and veterinary medicine. The emergence of AMR, especially among *Enterobacteriaceae* has been increasing day by day and poses serious threat for both human and animal health with limited antimicrobial therapeutic options (2).Among *Enterobacteriaceae, E. coli*is a major environment contaminant as it is natural inhabitant of GITmicrobiota of animal and human and plays important role in transmission of AMR through horizontal gene transfer. Unfortunately, AMR from the environmental sources has received less attention compared to that from the human and animal health spheres despite the significant variation in the geographical spread of AMR bacteria in the environment (3). Forces working behind the dissemination of antimicrobial resistantbacteria are uncountable but one commonly neglected source happens to be the wild avifauna living in close vicinity of animals. The cattle egret (*Bubulcus ibis*, a gregarious species of bird) which is found foraging commonly with grazing livestock, can acquire AMR bacteria through commensalism from environment. Since no work has been done in this area on cattle egrets, the present study was undertaken with aim to establish their role as a potential source ofAMR especially extended spectrum beta lactamase (ESBL) producing bacteria.

2.MATERIALS AND METHODS

Locale: The study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya. The samples werecollected from diverse places of university cattle farm by swab technique and brought to laboratory in ice box.

Sample Processing: Total 50 faecal swab samples were collected from contaminated environment of cattle farm and each sample was homogenized in 5ml of sterile normal saline solution. These suspensions were kept for 2 hours at room temperature to allow larger debris to settle down and bacterial-rich supernatant was utilized for subsequent analyses.

Isolation and Identification: The isolation and identification of *E. coli* was done as per the method of Edward and Ewing (4). All samples were enriched with 2ml nutrient broth and incubated at 37^0C for 24 hrs. A loopful inoculum was taken and streaked on MacConkey agar plates added with 2 mg/L cefotaxime, then incubated at 37^0 C for 24 hrs. Colonies showing rose pink colouration (Fig. 1) were streaked on Eosine Methylene Blue (EMB) agar plates and incubated at 37^0 C for 24 hrs. The greenish metallic sheen colonies (Fig.2) on EMB were tentatively considered as *E. coli*and taken onto sterilized nutrient agar slant.Further identification of isolates was done by Gram's staining, motility test and various biochemical tests *viz.* IMViC pattern, catalase test, nitrate reduction, urease test, triple sugar iron agar and sugar fermentation reaction etc.

Antibiotic Susceptibility Test: Bauer'sdisk diffusion method (5) was employed to determine the isolate's resistance or susceptibility to various antibiotics (Table 2). A uniform lawn of fresh 24 h grown culture of test organism (1.5 $x10^8$ organism/ml) was spread on Muller and Hinton Agar plates (MHA) and then commercially available discs were placed at 30 mm apart from centre on the surface of MHA plates and incubated at 37^0Cfor 24 hrs. The results were interpreted as per manufacturer's guidelines (Hi Media).

Phenotypic confirmation of ESBL producers: Double disc synergy test (DDST) was used for phenotypic confirmation of ESBL producers. All *E. coli* isolates were subjected to DDST using ESBL kit 1 (Cefotaxime, Cefotaxime/Clavulanic acid) and Kit 3 (Ceftazidime, Ceftazidime/Clavulanic acid) of Hi-media. A uniform lawn of fresh 24 h grown culture of test organism (1.5 $x10^8$ organism/ml) was prepared on Muller and Hinton Agar plates. The commercially available discs were placed at 30 mm apart from centre on the surface of plates and incubated at 37^0Cfor 24 hrs. The results were interpreted as per CLSI guidelines (6).

Molecular characterization of ESBL producers

The ESBL genes viz. bla_{TEM} and $bla_{CTX\text{-}M}$ were targeted for molecular characterization of ESBL producers using published primer sequences (Table1). The DNA templates were prepared using snap- chill method as described by Pandey *et al.*(7). The PCR assay was performed in 20 µl final volume comprising 10µl of master mix, 2µl of forward and reverse primer (100pmol), 2µl of MgCl2, 2µl of DNA template and 2 µl of nuclease free water. The PCR cycling condition included initial denaturation at 95^0C for 5 min followed by 35 cycles at 94^0C for 30 sec, annealing at 50^0C for 35 sec, elongation at 72^0 C for 40 sec and final extension at 72^0 C for 5 min. The amplified PCR products were run in 1.5% agarose gel and analysed using gel documentation system (Uvi tech, UK).

Table1: Primer sequence used for identification of ESBL genes

Gene	Sequence (5' 3') →	Product size	Reference
bla_{TEM}	F-ATGAGTATTCAACATTTCCG R-TTAATCAGTGAGGCACCTAT	851bp	Grimm *et al.* (8)
bla_{CTX}	F-CGCTTTGCGATGTGCAG R-ACCGCGATATCGTTGGT	551bp	Paterson *et al.*(9)

Multiple Antibiotic Resistance (MAR) Index: MAR index is the number of antibiotics to which test isolate displayed resistance divided by the total number of antibiotics to which the test organism has been evaluated for sensitivity. MAR index of each isolate was calculated as per the method of Krumpermann (10).

Congo red binding test: The Congo red binding (CR)test was carried out as per the technique of Berkhoff and Vinal (11). The isolates were streaked on the plate of Trypticase Soya Agar (TSA) containing 0.03% Congo red dye and incubated at 37^0 C for 24-72 hr. Appearance of brick red coloured colonies within 24 to 72 h was recorded as a positive reaction, while the colonies that didn't bind dye and remained white or grey even after 72 h PI, were considered as negative.

Haemolysis test:*E. coli*isolates were also tested for haemolysin production by plate as well as tube inoculation method. Enterohaemolysin production on plates was determined as per the method of Joshi and Joshi (12). The isolates were cultivated in blood agar base supplemented with 5% w/v sheep RBCs, 10mM calcium chloride and incubated at 37^0 C for overnight. Haemolysin production was indicated by the zone of complete lysis of erythrocyte and clearing of medium. The tube method used for alpha-haemolysin production was a slight modification of those of Asnani *et al.* (13). Isolates were inoculated in 1ml of sterilized nutrient broth supplemented with equal volume of washed sheep RBCs (5% V/V) in graduated centrifuge tube. The tubes were incubated at 37^0 C for 4-6 hrs with intermittent agitation followed by centrifugation at 6000 rpm for 5 min. All the test cultures were observed for transparent reddish colour of supernatant; the signs of haemolysis.

3. Results and discussion:

Antimicrobial resistance is a multifaceted global health problem and its containment requires a multi-pronged approach like One Health concept. This concept rightly recognizes the interrelatedness of human, animal and environment health and fosters collaborative and multisectoral efforts to tackle the challenge (3). The present study was conducted with aim to establish the role of egret found in cattle farm as potential spreader of Multi Drug Resistant (MDR) bacteria. Keeping in view, total 50 faecal droppings of cattle egret were collected from the environment and processed in the laboratory for isolation and identification of *E.*

coli. On the basis of morphological, growth and biochemical characteristics, 20 isolates (66.66%) were identified as *E. coli*out of 45 isolates recovered from faecal swabs. These findingswere found in agreement with the reports of previous workers (**7,** 15, 16). All *E. coli* isolates were subjected to antibiogram study(Fig.3) against 11 antibiotics viz. Ampicillin (AMP), Cloxacillin (COX), Norfloxacin (NX), Kanamycin (K), Streptomycin (S), Gentamycin (GEN), Polymyxin B, Triple Sulpha (S3), Cefotaxime (CTX) and Imipenem (IMP). The results ofantibiotic susceptibility test(ABST) are mentioned in Table2. In this study, all *E. coli* isolates were found resistant to Ampicillin (100%), Cloxacillin (100%)and Cefotaxime (100%) followed by Polymyxin (45%), Kanamycin (33%), Norfloxacin (22%). Merely two isolates exhibited resistance for Streptomycin (10%) and Trimethoprim (10%) (Fig.7). Similar findings were reported by Yadav*et al.* (**16**) for *E. coli* of bovine origin against cefotaxime, ampicillin, norfloxacin and trimethoprim from the same area. It reveals that sharing of environment could result in transmission of AMR bacteria in wild bird also. Antibiotics like Gentamycin, Triple Sulpha and Imipenem were found sensitive against all isolates (100%) followed by Streptomycin (90%) and Trimethoprim whereas Norfloxacin (55%), Polymyxin (55%) and Kanamycin (30 %) were found moderately sensitive.Likewise, Hasan (**17**) also recorded highest resistance for Cloxacillin, moderate resistance for Kanamycin, and highest sensitivity for Imipenem for *E. coli* isolated from UTI patients. The Multi antimicrobial resistance index of all the isolates was found to be more than 0.27 in this study (Table 3). MARI ≥0.2 indicates that pathogen is from a "high risk environment" where antibiotics are being used indiscriminately without antibiogram study (**10**).In similar study, Ahmed and Gulhan (**18**) also reported occurrence of MDR *E. coli*in Gulls and Pigeon showing a varying degree of resistance to Tetracycline, Kanamycin, Streptomycin, Ampicillin, Chloramphenicol, Trimethoprim, Gentamicin, Enrofloxacin and Ciprofloxacin.

Table 2: Antimicrobial resistance pattern of *E. coli* isolates of Cattle Egret

S. No.	Antibiotic	Disc Conc.	Number of *E. coli*Isolates (%)		
			Resistant	Intermediate Sensitive	Sensitive
1.	Ampicillin (AMP)	10 mcg	20(100)	-	-
2.	Cloxacillin (COX)	10 mcg	20(100)	-	-
3.	Norfloxacin (NX)	10 mcg	5 (22)	4 (20)	11 (55.0)
4.	Kanamycin (K)	30 mcg	6 (30)	8 (40)	6 (30)
5.	Streptomycin (S)	10 mcg	2 (10)	-	18 (90)

6.	Gentamycin (GEN)	10 mcg	-	-	20 (100)
7.	Polymyxin B (PB)	300 unit	9 (45)	-	11(55.0)
8.	Triple Sulpha (S3)	300 mcg	-	-	20 (100)
9.	Trimethoprim (TR)	10mcg	2 (10)	-	18 (90)
10.	Cefotaxime (CTX)	30mcg	20(100)	-	-
11.	Imipenem (IMP)	10 mcg	-	-	20(100)

Table 3: MultiAntimicrobial ResistanceIndex(MARI) of *E. coli* isolatesof Cattle Egret

Isolates	AMP	COX	NX	K	S	GEN	PB	S3	TR	CTX	IMP	MARI
E1Aa	R	R	S	S	S	S	R	S	S	R	S	0.36
E1	R	R	S	S	S	S	S	S	S	R	S	0.27
E2	R	R	S	S	S	S	S	S	R	R	S	0.36
E1Ab	R	R	R	S	S	S	R	S	S	R	S	0.45
E3	R	R	S	S	S	S	S	S	S	R	S	0.27
E1Ba	R	R	IS	R	S	S	R	S	S	R	S	0.45
E4	R	R	IS	R	S	S	S	S	S	R	S	0.36
E1Bb	R	R	S	IS	S	S	R	S	S	R	S	0.36
E5	R	R	S	IS	S	S	S	S	S	R	S	0.27
E12a	R	R	R	IS	S	S	R	S	S	R	S	0.45
E6	R	R	R	IS	S	S	S	S	S	R	S	0.36
E12b	R	R	IS	IS	S	S	R	S	S	R	S	0.45
E7	R	R	IS	IS	S	S	S	S	S	R	S	0.27
E1Bb1	R	R	S	R	S	S	R	S	S	R	S	0.45
E8	R	R	S	R	S	S	S	S	S	R	S	0.36
E1Bb2	R	R	S	IS	R	S	R	S	S	R	S	0.45
E9	R	R	S	IS	R	S	S	S	S	R	S	0.36
E12a1	R	R	R	R	S	S	R	S	S	R	S	0.54
E10	R	R	R	R	S	S	S	S	S	R	S	0.54
E11	R	R	S	S	S	S	S	S	R	R	S	0.36

Note: S= sensitive, IS= Intermediate sensitive, R= Resistant

All *E. coli* isolates were also assessed for their propensity to produce ESBL using Cefotaxim and Ceftazidime and 18 isolates (91%) were screened out to be ESBL producers (**6)**. In phenotypic testing, 12 (60 %) isolates were confirmed as ESBL positive by DDST (Fig. 4), which coincided with the observations of Vibha *et al.* (**19**). Gene distribution study of phenotypically positive ESBL isolates revealed that $bla_{CTX}$was presentin 12 (60 %) isolates (Fig. 5), while $bla_{TEM}$was detected in 8 (40 %) isolates (Fig.6). Previous studies conducted in this area of Eastern UP have also shown predominance of bla_{CTX} gene (**15,19, 20**). There was little difference in the prevalence between phenotypic and genotypic method and these

findings corroborated with the observation of Vibha *et al.* (**19**), Prajapati*et al*. (**20**) and Badri *et al.* (**21**).

The *E. coli* isolates fromcattle egrets were further tested for pathogenicity traits by C R binding test and haemolytic assay. In this study, C R binding activity was revealed by 10 isolates (50%) showing brick red colonies. This marker can differentiate pathogenic strains from commensal ones and has been used as epidemiological marker of APEC by some researchers. Binding of Congo Red is associated with presence of virulence genes such as *ompA*, *iss*and *fimH*and genes for multiple resistance to antibiotics as reported by Abdel *et al.* (**22**). Haemolytic activity was exhibited by 8 isolates (40%) by both plate as well as tube method. This finding was in agreement with the observation of Pandey *et al.* (**7**). All these HA and CR positive isolates were considered as enteropathogenic. Both of the tests have been used previously by various researchers (**12, 22**, **23**) to discriminate invasive and non-invasive strain of *E. coli* of bovine and poultry origin.

This study illustrated that antibiotics are widely used in the treatment of human and animal infection leading to proliferation of multi-drug resistant (MDR) bacteria which are distributed in environments. The polluted environment serves as source of antimicrobial-resistant (AMR) bacteria for wild birds or migratory birds which acts as potential spreaders of resistant elements through migration.

4. Conclusion

In this study, occurrence of MDR positive *E. coli*with MARI ≥ 0.2 from cattle egrets poses a serious risk to human and animal health by contaminating the environment with their faeces. It shows that antibiotics are being used indiscriminately both in human and animal healthcare system leading to the contamination of environment. These cattle egret could play a dynamic role in the transmission of AMR bacteria from one place to another place. Hence, a largescale epidemiological study is needed to determine possible transmission of AMR bacteria between wild bird, environment, animal and human for investigating genetic relationship between the strain from one health point of view.

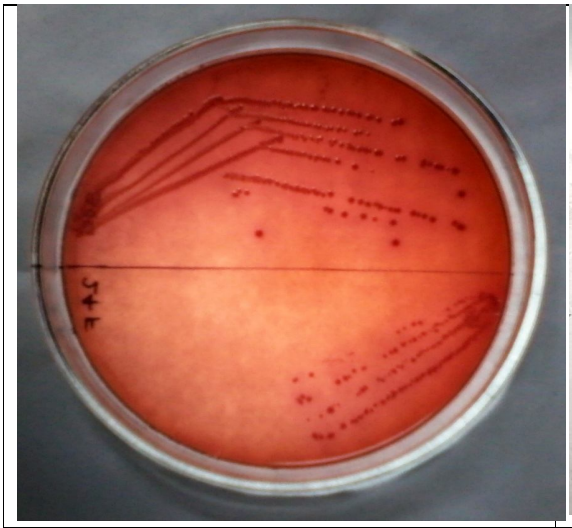

Fig.1 Rose Pink colony of *E. coli* on MLA

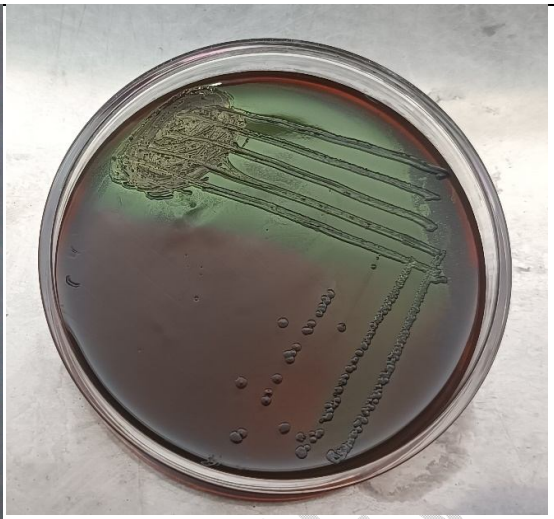

Fig.2 Metallic sheen colony of *E. coli* on EMB

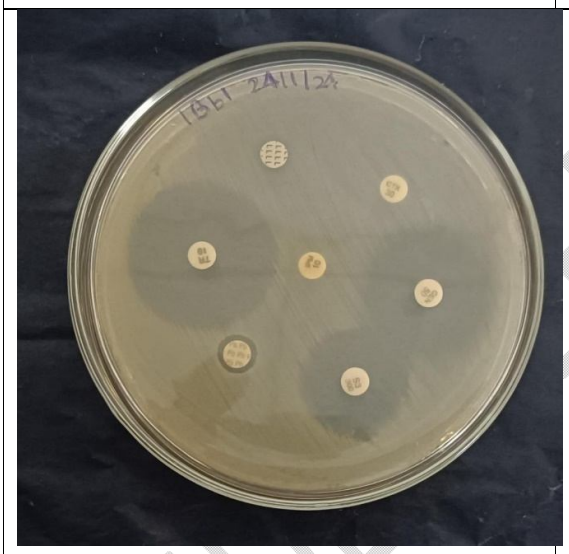

Fig. 3 AMR screening of *E. coli*by disc diffusion test

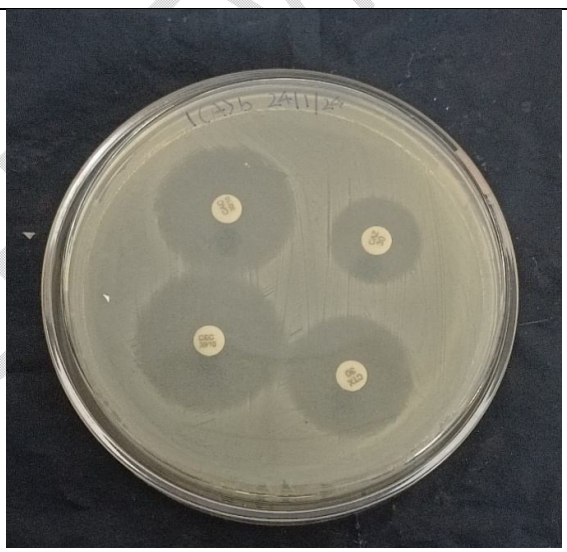

Fig. 4 Double Disc Synergy Test for phenotypic confirmation of ESBL

Fig. 5PCR amplification of $bla_{\text{CTX-M}}$ gene (551bp)

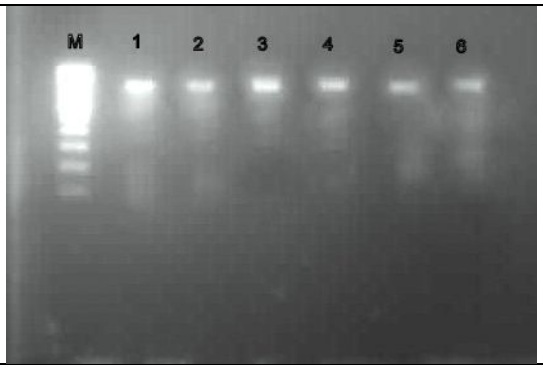

Fig. 6PCR amplification of bla_{TEM} gene (851bp)

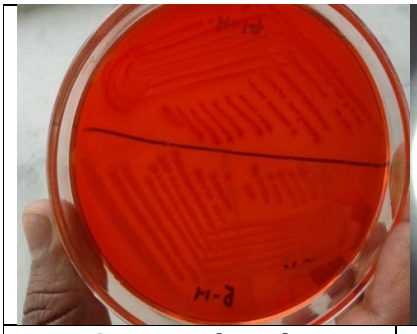

Fig.7 Congo Red Binding test

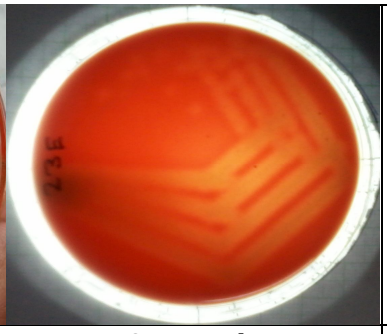

Fig.8 Haemolysis (Plate Method)

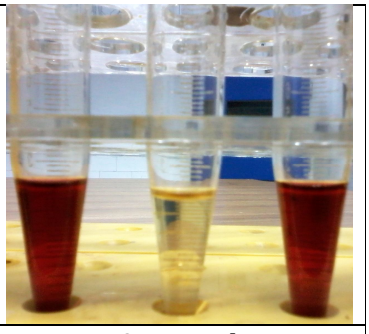

Fig.9 Haemolysis (Tube Method)

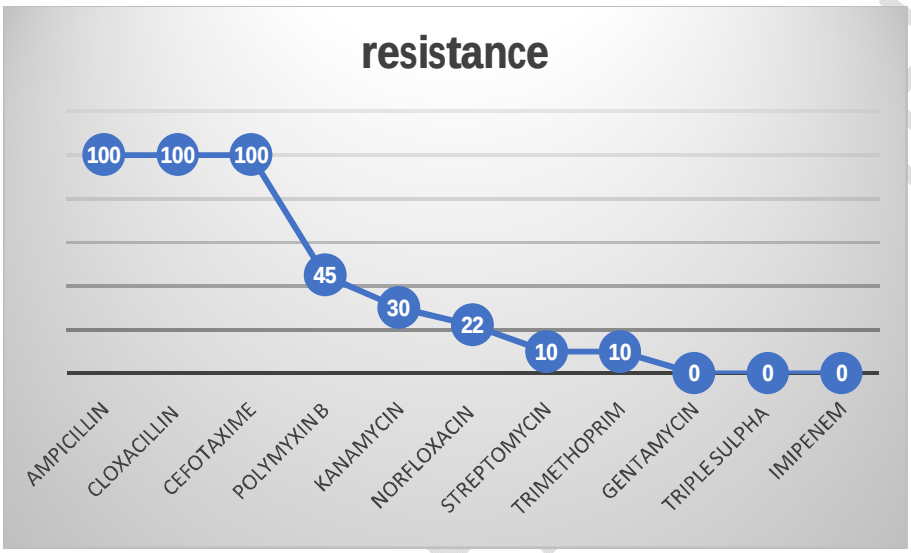

Fig 10. AMR profile of *E. coli* isolated from cattle egrets

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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