Research Article

In vitro and *in vivo* evaluation of antimicrobials in *Escherichia coli* infection in broilers and evaluation of ciprofloxacin in induced colibacillosis

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Abstract

The present study was conducted to 1) Survey and record the data regarding use of antimicrobials in *Escherichia coli* infection in broiler poultry flocks 2) Evaluate the sensitivity of *E. coli* against antibiotics and 3) Evaluate the ciprofloxacin in induced colibacillosis. For this purpose, fifty (50) broiler poultry farms were surveyed and *in vitro* evaluation of antimicrobials in *E. coli* infection was evaluated. The farmer level data regarding awareness about antibiotic usage pattern, storage of antibiotics, withdrawal period and antibiotic resistance was evaluated. Data revealed that most of broiler poultry farmers relied on quinolones (especially ciprofloxacin) to combat *E. coli* infection. The morbid samples from the *E. coli* infected farms were collected, pathogenicity of *E. coli* was checked and sensitivity against seven antibiotics viz. ciprofloxacin, enrofloxacin, gentamicin, oxytetracycline, colistin, neomycin and amoxycillin was evaluated. Sensitivity tests showed *E. coli* was highly sensitive against ciprofloxacin, enrofloxacin, and gentamicin. On the basis of antibiogram, the efficacy of ciprofloxacin was determined under *in vivo* conditions. Accordingly, 150 Hubbard (un-sexed) broiler chicks were divided into three groups viz. A, B & C each having 50 chicks; group A was kept as uninfected and untreated (control), group B was experimentally infected and treated with ciprofloxacin while group C was infected and kept untreated. The mortality in group A, B and C was recorded as 2, 14 & 28 %, respectively. The cumulative feed consumption in group A, B and C was 84, 87 & 86 kg respectively. Mean weight gain in group A, B and C was 882.47, 868.70 & 801.29 gm, respectively. The FCR in group A, B and C was 1.9, 2 and 2.15, respectively. It was concluded that the use of ciprofloxacin had high efficacy against *E. coli* infection in broiler chicks.

Keywords: Antimicrobials; Broilers; Ciprofloxacin; *Escherichia coli*

Introduction

Poultry farming is one of the most profitable businesses in Pakistan. Poultry sector is the second largest industry in Pakistan after textile. It has emerged as a cheap and economical substitute for beef and mutton in Pakistan. Its importance can be judged from the fact that almost every family in rural areas and every fifth family in urban areas is associated with poultry production activities in one way or the other. The poultry industry has developed rapidly over the last three decades with an impressive growth rate of over 8% [1]. The development, expansion and intensification of poultry has faced problems of disease, nutrition and management. Colibacillosis caused by *E. coli* is a disease of economic concern in poultry industry [2-4]. *E. coli* is responsible for a variety of poultry diseases as it comprises of non-pathogenic and pathogenic strains that cause a number of diseases in host species including colibacillosis, colisepticemia, Hjar's disease, coligranuloma, peritonitis, salpingitis, synovitis, omphalitis and air sac disease [5]. *E. coli* is also responsible for high chick mortality, air sacculitis, pericarditis and may predispose bird to adenovirus, reovirus, coronavirus and IBD virus and other secondary bacterial infection [6].

An incidence of 52% of colibacillosis is reported through isolation and identification of *E. coli* from trachea, lung, heart, blood, liver and spleen of poultry birds [7]. The prevalence of colibacillosis of 1% and 0.5% in 25-30 days old and 31-35 days old broilers, respectively is observed [8].

Antimicrobial therapy is an important tool in reducing the losses due to incidence and mortality associated with avian colibacillosis [9, 10, 11]. The fluoro-quinolones are a class of antimicrobials that exhibit excellent activity against gram-negative organisms [12]. Among different fluoro-quinolones ciprofloxacin is of the potential interest for veterinary medicine. It has a wide antibacterial spectrum against

mycoplasma, salmonella, *E. coli*, *Haemophilus paragallinarum*, *Pasteurella multocida* and staphylococcal infections in chicks [13, 14]. Quinolones are bactericidal and act by inhibiting bacterial DNA replication and transcription. To facilitate coiling, winding and unwinding, the DNA gyrase allows the stands to be cut and reconnect. DNA gyrase, a topoisomerase, consists of A and B subunits. The most common target site for quinolones is the A-subunit of DNA gyrase coded by the gene *gyrA*. In Pakistan, the efficacy of this drug has not been evaluated. The present study was designed to determine *in vitro* and *in vivo* efficacy of ciprofloxacin in broiler chicks against *E. coli*.

Materials and Methods

Survey for *E. coli* infected farms

Fifty (50) broiler farms located in district Toba Tek Singh were visited for collection of data. Toba Tek Singh is located between 30°33' to 31°2' degree north latitudes and 72°08' to 72°48' degree longitudes. Data regarding drinking water, feeding, vaccination, disease-related morbidity, mortality and antibiotic usage, duration, efficacy, storage, brand, price and with drawl period were collected with an emphasis on *E. coli* infection. The efficacy of ciprofloxacin medication was determined.

Isolation of *E. coli*

The livers and hearts of sick/dead chicks suffering from *E. coli* infection were collected. Samples were transported to the laboratory and processed as soon as possible for the isolation of *E. coli*. The MacConkey's agar medium, triple sugar iron agar medium and Simon's citrate medium were used for isolation, purification and identification of the organism [15]. The morphological, cultural staining characteristics, sugar fermentation, biochemical properties of isolates were determined. Biochemical tests were conducted according to recommended procedures [15-17].

Pathogenicity of *E. coli*

A gut loop assay was performed for the determination of pathogenicity [18]. The rabbits were anaesthetized with ketamine HCl and ileum was exposed. ½ mL culture of the test isolate was delivered into the lumen of the intestine. An equal volume of normal saline was injected into the intestine of control rabbits. The cavity was sutured with catgut and animals were kept under extreme care and observations were recorded.

Antibiotic sensitivity of *E. coli*

The susceptibility of pathogenic isolates to seven different antibiotics was determined using the disc diffusion method [19]. Mueller-Hinton agar was used for antibiotic susceptibility testing. Nutrient agar was incubated at 37°C for 15 hrs. The growth was diluted with normal saline to a density visually equal to 0.5 Mc-Farland. Using a

sterile swab, the bacterial suspension was spread in 3 different plates on Mueller-Hinton agar. The plates were dried at room temperature for 5 minutes. Seven commercially available antibiotic discs i.e. amoxicillin, gentamicin, ciprofloxacin, oxytetracycline, colistin, enrofloxacin and neomycin were placed on the agar plates using sterile forceps. The plates were incubated at 37°C for overnight. The radius of zone of inhibition of each antibiotic was measured using Schering antibiotic zone gauge and interpreted according to the zone size interpretative chart. *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection) was used as a quality control organism. The resistant, intermediately susceptible and fully susceptible isolates to each antibiotic were measured and recorded (Table 1).

Table 1. Disc potencies of antibiotics, inhibition zone radius (mm) to categorize *E. coli* and inhibition zone size of quality control organism (*S. aureus* ATCC 25923)

Name of Antibiotic	Symbol	Disc Potency (µg / disc)	Inhibition zone radius (mm) to categorize <i>E. coli</i> & interpretation			Inhibition zone size (mm) of quality control organism (<i>S. aureus</i> ATCC 25923)
			Susceptible	Intermediate	Resistant	
Ciprofloxacin	Cip	10	≥21	16-20	≤ 15	
Enrofloxacin	Enr	5	≥21	16-20	≤15	20-24
Gentamycin	G	10	≥15	13-14	≤12	19-27
Colistin	Col	25	≥16	13-14	≤13	27-30
Oxytetracycline	Oxy	30	≥18	13-15	≤14	12-15
Amoxicillin	Am	25	≥18	14-17	≤13	28-36
Neomycin	N	25	≥17	13-16	≤12	18-26

National Committee for Clinical Laboratory Standards (NCCL, 1990)

Test culture

A pathogenic isolate of *E. coli* was inoculated into the fresh nutrient broth and incubated at 37°C for overnight. The viable bacteria were counted [15]. The broth was diluted with phosphate buffer saline (PBS) to have approximately 1×10^6 CFU organisms / mL, which was used for experimental infection.

Evaluation of ciprofloxacin in induced *E. coli* infection

For evaluation of ciprofloxacin in induced *E. coli* infection one hundred and fifty (150) day-old Hubbard (un-sexed) broiler chicks were reared under uniform conditions of management and nutrition at department of Veterinary Clinical Medicine and Surgery, University of Agriculture, Faisalabad. All chicks were vaccinated against Newcastle

disease, Infectious bursal disease (IBD / Gumboro) and Hydropericardium syndrome (Table 2). At 20th day of age, chicks were randomly divided into three equal groups viz. A, B and C. 1mL of diluted broth culture of a pathogenic strain of *E. coli* (1×10^6 CFU organisms / mL) was inoculated orally with the help of the crop tube to the chicks of group B and C, while the chicks of group A were kept as control (uninfected & untreated). All the chicks were kept under observation for development of clinical signs and

symptoms for 2 weeks. After the appearance of clinical signs (dullness, depression, vent pasting, respiratory and enteric signs) and postmortem lesions (pericarditis, perihepatitis and air sacculitis), the chicks of group B were treated with ciprofloxacin 20% @ 1 ml / 4L (Ciprosel®) PO as recommended by the manufacturer for 5 days. Chicks in group C were challenged and untreated control, while group A was control (unchallenged and untreated) (Table 3).

Table 2. Vaccination schedule of experimental chicks

Age (Days)	Vaccine	Route
4	ND (1 st dose)	E/D
9	Gumboro (1 st dose)	E/D
15	Hydropericardium Syndrome	S/C
19	Gumboro (2 nd dose)	D/W
23	ND (2 nd dose)	D/W

E/D Eye drop; S/C Sub-cutaneous; D/W Drinking water

Table 3. Different treatments in experimental groups

Treatments	Group A	Group B	Group C
No. of chicks	50	50	50
Quantity of inoculums	1mL	1mL	1mL
Concentration of inoculums	-	1×10^6 CFU / bird	1×10^6 CFU / bird
Antibiotic usage	-	Single	-
Treatment (Antibiotic usage)	-	Ciproflaxocin 20%	-
Concentration of drug	-	20gm / 100 mL	-
Dose rate (@)	-	1 mL / 4 L	-
Route of Treatment	-	Oral / Drinking Water	-
Name of Brand	-	Ciprosel ®	-
Duration of Treatment (Days)	5	5	5

Parameters of the efficacy of treatment

Parameters of mortality, cumulative feed consumption, mean body weight and feed conversion ratio were observed in experimental groups for the determination of drug efficacy.

Statistical analysis

Data was statistically analyzed / compared accordingly.

Results and Discussion

Avian colibacillosis is one of the major endemic diseases affecting poultry industry

worldwide [3] and is main cause of economic losses [2]. Avian pathogenic *E. coli* (APEC) causes extensive mortality in poultry flocks [4] and may pose a human health risk [2].

Fluoro-quinolone is a group of bactericidal antibiotics, has large volume of distribution, wide spectrum of activity and favourable margin of safety. No plasmid related resistance is reported [20, 21]. Several members of this group i.e. flumequine, norfloxacin, ciprofloxacin,

enrofloxacin are used in poultry for the treatment of different infections [14, 22, 23]. Ciprofloxacin was mainly developed for veterinary use against mycoplasmosis and colibacillosis.

Survey report regarding treatment of *E. coli* infection in broiler birds is shown in (Table 4). Frequency distribution (range of poultry farmers) of awareness about antimicrobial therapy is shown in (Table 5).

Table 4. Survey for the treatment of *E. coli* infections

Flock size	Mortality		<i>E. coli</i> infection (Primary / secondary)	Percent reduction	Medication
	Pre-treatment	Post-treatment			
3200	300	65	Secondary	7.34	Ciprosel
3000	140	25	-do-	3.83	VetyEnrox 20%
2000	50	15	-do-	1.75	Atiquine 20%
2500	200	120	-do-	3.2	VetyEnrox 20%
3800	296	90	-do-	5.43	Ciprosel
3500	450	125	-do-	11.28	Ciprosel
2000	158	80	-do-	3.25	Flumiquine 20%
2800	390	127	-do-	9.39	Tribriksen + CTC 20%
5000	305	90	-do-	4.43	Flumesol 50%
2500	400	290	-do-	4.4	Colisol powder
2800	510	235	-do-	9.82	Ciprosel
10000	250	52	-do-	2	Floxamycin
4000	398	85	-do-	7.85	Enrosol S + Colimycin
2200	290	108	-do-	8.27	Ciprosel
4000	311	105	-do-	5.15	Ciprosel
2500	205	180	-do-	5.46	Tribriksen
3200	320	100	-do-	6.87	Ciprosel
2500	150	69	-do-	3.24	Floxamycin
5500	450	125	-do-	5.90	Enrosol S
3100	220	105	-do-	3.7	Atiquine 20%
6000	440	135	-do-	5.08	VetyEnrox 10%
2900	297	85	-do-	7.41	Ciprosel
3000	189	50	-do-	4.63	Oramox Plus
3000	120	18	-do-	3.34	TylanSolube
4000	250	20	-do-	5.75	Norfloxx 20%
2700	185	130	-do-	2.03	Atiquine 20%
7500	492	90	-do-	5.36	Quintox
4200	435	80	-do-	8.45	Ciprosel
3500	320	100	-do-	6.28	Norfloxx 20%
2700	189	50	-do-	5.14	Enrosol S
2200	140	32	-do-	4.90	Tribriksen
4500	312	95	-do-	4.82	Colisole powder
2800	142	63	-do-	2.82	Oxytet
1500	90	33	Primary	3.80	Oxytet + colimycine
3000	110	45	Secondary	2.16	Ateuine-P 50
3500	319	115	-do-	5.82	Ciprosel
1800	120	55	-do-	3.61	Oramox Plus

2000	115	43	-do-	3.60	Tylan Soluble
2500	297	87	-do-	8.40	Ciprosel
3200	180	65	-do-	3.59	Floxamycin
3500	315	125	-do-	5.42	Ciprosel
4000	365	138	-do-	5.67	Ciprosel
1400	70	25	-do-	3.21	Tribrissen
1000	32	8	-do-	2.40	Neomycin Sulphate
1200	28	6	-do-	1.83	Flumesol
1800	44	13	-do-	1.72	Flumesol
3200	115	42	-do-	2.28	Oxytet
1700	72	25	-do-	2.76	Colisol + Oxytet
2000	110	45	-do-	3.25	Oramox Plus
5200	415	120	-do-	5.67	Enrosol S

Table 5. Frequency distribution (range of poultry farmers) of awareness about antimicrobial therapy

Parameters		Range of poultry farmers
Antimicrobial source	Distributor	36 – 40
	Veterinary medical store	0 – 5
	Other	0 – 5
Prescriber of antimicrobial therapy	Distributor	31 – 35
	Self	0 – 5
	Veterinarian	6 – 10
Dosage		16 – 20
Withdrawal period		0 – 5
Price of antibiotic		36 – 40
Proper storage at farm		0 – 5
In-effectiveness of antimicrobials (particular antibiotic)		6 – 10
Resistance to antimicrobials		0 – 5
Irrational use		36 – 40

***In vitro* characteristics of *E. coli* isolates**

In our study the morphological and biochemical characteristics were observed for isolation and identification of *E. coli* isolate. After 24 hrs of incubation at 37°C on MacConkey's agar all *E. coli* isolates developed pink, pin pointed colonies, having luxuriant growth and colony size varied from 2–3 mm in diameter. The isolates were cocco-bacillary, long filamentous, gram-negative, non-spore forming and motile. On the basis of triple sugar iron agar reaction, the isolates fermented lactose and sucrose with the production of gas. The isolates on Simon's citrate medium changed the colour from green to blue.

Biochemical reactions of *E. coli* isolates included lactose and sucrose fermentation with the production of gas, but non-

production of hydrogen sulphide. On Simon's citrate medium, the isolates changed the colour of the medium from original green to blue. Other researchers also observed the bio-chemical characteristics of *E. coli* strains with same method [18, 26]. The microscopic, cultural and morphological characteristics of *E. coli* of present study were in-consistent with results of previous studies [24, 25, 27].

***In vitro* antibiotic susceptibility of *E. coli* isolates**

Antibiotic susceptibility profiles of *E. coli* indicated ciprofloxacin and enrofloxacin were 100% effective, while effectiveness of gentamicin, neomycin, colistin, oxytetracycline and amoxicillin were 83, 69, 62, 55 and 45%, respectively (Table 6). Previously sensitivity of 83.08% to both ciprofloxacin and Enrofloxacin against *E.*

coli is noticed [28]. The high sensitivity of 100% of *E. coli* to ciprofloxacin is also recorded [24].

The use of antibiotics and other drugs for the control and treatment of colisepticemia helps to avoid economic losses from the disease. Most of the *E. coli* strains are normally sensitive to most of the antibiotics and chemo-therapeutic agents but in recent years' resistance has been encountered in many cases. Therefore, it is advisable to perform an antibiotic sensitivity test to minimize the hazards of drug resistance and to avoid economic losses on treatment [29]. In the present study the antibiotic sensitivity test of *E. coli* isolates was performed using seven antimicrobial agents. The *E. coli* isolates were most sensitive to ciprofloxacin and enrofloxacin followed by gentamycin and Colistin and resistant to amoxicillin, neomycin, and oxytetracycline. In other studies, the antibiotic sensitivity of *E. coli* isolates against flumequine was most effective [30]. Isolates having septicemia have also shown sensitivity to nalidixic acid [31]. Less susceptibility *E. coli* against antibiotics has also been recorded [29, 32, 33]. It is also reported that *E. coli* of poultry are resistant to one or more antibiotics. It is advisable to determine the drug sensitivity of the strains of *E. coli* involved in the diseased condition. Researchers have reported susceptibility and resistance of *E. coli* strains to various antibacterial agents [33-36]. The variance in drug susceptibility might be due to prevalent serotypes and frequent use of antibacterial in the feed or drinking water. In our study disc diffusion

test was used to determine susceptibility to antibiotics. The disc diffusion method is also used for antibiotic susceptibility profiles, resistance and multiple drug resistance to *E. coli* by other researchers [24, 37].

There is widespread antimicrobial resistance to avian pathogenic *E. coli* (APEC) [38]. Also, there is emerging drug resistance to *E. coli* associated with colibacillosis [24]. Studies on antibiogram profile of *E. coli* isolate of chicken showed that it is also multidrug resistant [8]. Resistance to APEC isolates against antimicrobials has increased over time and further improvements in treatment guidelines against bacterial pathogens of poultry are required [39]. Resistance of 30.55% of *E. coli* to ciprofloxacin is reported [40]. The resistance of 46.8% of APEC isolates to ciprofloxacin is reported due to the carrying of resistance genes [4]. 72% antimicrobial resistance [41] and further higher antimicrobial resistance of 87% against ciprofloxacin is also reported [42]. The reason for multidrug resistance in APEC may be due to the heavy usage of antibiotics for the treatment of diseases in poultry flocks [42]. The other significant risk factor associated with the presence of multiple drug resistant (MDR) APEC is the use of ground water and closely located poultry farms [38]. As *E. coli* are part of normal flora of birds, the prevention and control of colibacillosis can be challenging [43]. The antibiogram profile of the *E. coli* isolate can help in therapeutic decision-making in the treatment of colibacillosis [25].

Table 6. *In vitro* susceptibility of *E. coli* to antibiotics

Antibiotics	No. of samples	Susceptibility (%)		
		Susceptible	Intermediate	Resistant
Ciprofloxacin	100	100	-	-
Enrofloxacin	100	100	-	-
Gentamycin	100	83	-	17
Neomycin	100	69	-	31
Colistin	100	62	11	27
Oxytetracyclin	100	55	15	30
Amoxicillin	100	45	-	55

Pathogenicity of *E. coli*

In nutrient broth *E. coli* isolates produced slight pellicle formation at 18–24 hrs with a slimy deposit at the bottom of the tube which on shaking produced a uniform turbidity. The identified culture of *E. coli* was tested by gut loop ligation method for pathogenicity of the strain. In the positive cases intestinal part became triple size as compared to the normal. The fluid accumulation in intestine indicated positive results, whereas, absence of fluid accumulation indicated negative results. Along with the presence of fluid, the amount of the fluid also served as an indicator of positive results. The higher the amount of fluid observed, the higher the pathogenic strain of *E. coli* it represents. The culture that showed maximum pathogenicity was selected for induced *E. coli* infection. The control sample was inoculated with normal saline instead of *E. coli*. In the present study intestinal loop ligation method positive (i.e., pathogenic) *E. coli* were identified by increased size (i.e. triple fold than normal) and increased luminal fluid of the intestine, which coincide with the results of previous studies [18, 23, 26].

In vivo efficacy of ciprofloxacin in induced *E. coli* infection

In our study the artificial infection of colibacillosis was done using pathogenic isolate of *E. coli* which was inoculated @ 1×10^6 CFU organisms / mL through oral route in group B and C. The chicks were kept under observation for further two week. While in other studies the chicks were artificially infected with 3×10^8 CFU / 0.25mL of entero-toxigenic *E. coli* (ETEC) through intra-peritoneal route and infection developed within twelve hours [44]. In our study the chicks of group B were treated with ciprofloxacin 20% @ 1 ml / 4L (Ciprosel®) PO as recommended by the manufacturer for 5 days. Chicks in group C were challenged and untreated control, while group A was control (unchallenged and untreated)

Previously the efficacy of quinolones on *E. coli*, salmonella and mycoplasma infection had also been evaluated [45, 46]. In the present study, chicks that manifested clinical signs of colibacillosis were treated with Ciprosel® (ciprofloxacin 20%) for 5 days at the dose of 1 mL / 4L DW. The results of present study are in agreement with previous findings. The high *in vitro* and *in vivo* efficacy of ciprofloxacin may be attributed to its wide spectrum activity, large volume of distribution and high efficacy rate against *E. coli*, thus resulting in increased introduction for field use [20]. No plasmid resistance has been demonstrated and they have a factorable margin of safety [21]. The findings of high efficacy of ciprofloxacin in *E. coli* infection has also been previously reported [32, 33, 45, 46].

Mortality post-treatment

The mortality in group A, B and C was 1, 7 and 14 chicks, respectively after treatment (Table 7).

Postmortem findings

The post-mortem examination of chicks died in different experimental groups was performed. Thickened and cloudy air sacs were observed. In severe cases, caseous exudates on air sacs were present. Adhesive pericarditis and fibrinous perihepatitis were present in most of the cases. Enteritis along with excessive mucus was common. The primary lesions of colisepticemia were pericarditis, perihepatitis and air sacculitis were observed. Pericardial fluid became progressively more fibrinous. Necrotic foci in the heart muscles were present in chicks that succumbed to infection. The pericardial sac was thickened with pale-colored gelatinous exudates. Congestion of liver, spleen, kidney and small intestine were also observed. Similar findings were also observed in previous studies [14, 23].

The cumulative feed consumption, per bird feed consumption, mean body weight of chick and FCR in each group is presented in (Table 8). FCR of broiler chicks kept in the group A, B, and C were 1.90, 2.00 and 2.15 respectively. FCR increased in group

C because chicks in this group were infected with *E. coli* but were not treated.

Table 7. Post inoculation mortality pattern in groups

Age (days)	Day post inoculation (DPI)	Mortality			Total
		A	B	C	
21 st	1 st	-	4	4	8
22 nd	2 nd	-	2	4	6
23 rd	3 rd	-	1	3	4
24 th	4 th	-	-	1	1
25 th	5 th	-	-	1	1
26 th	6 th	-	-	-	-
27 th	7 th	-	-	-	-
28 th	8 th	-	-	-	-
29 th	9 th	1	-	1	2
Total =		1	7	14	22
Percentage (%) =		2	14	28	44

Table 8. Cumulative feed consumption, per bird feed consumption, mean body weight of chicks and FCR in groups

Age (days)	Total feed consumption			Feed consumption (kg) per bird			Mean body weight			Feed conversion ratio		
	A	B	C	A	B	C	A	B	C	A	B	C
17 th	23	23.50	23	0.46	0.47	0.46	282.29	288.58	284.94	1.63	1.63	1.63
23 rd	37	37	36.5	0.74	0.74	0.73	423.41	420.17	409.64	1.75	1.76	1.79
26 th	42	42.50	42.5	0.84	0.85	0.85	460.11	464.29	436.17	1.82	1.83	1.95
31 st	84	87	86	1.68	1.74	1.72	882.47	868.70	801.29	1.90	2.00	2.15

Conclusion

Use of ciprofloxacin in *E. coli* infection decreased mortality, increased feed consumption, increased body weight and increased FCR in broiler flocks. It was concluded that ciprofloxacin demonstrated very high efficacy against avian *E. coli* infection

Authors' contributions

Conceived and designed the experiments: T Ahmad, Performed the experiments: M Fiaz, Analyzed the data: T Ahmad, M Fiaz & A Sharif, Contributed materials/analysis/ tools: M Nadeem, SU Rehman, M Rizwan & M Umer, Wrote the paper: T Ahmad, M Fiaz, A Sharif & M Nadeem.

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