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Evaluation of Colicin Effect on the Induction of Treated Mice in Prevention of Infection Caused by *Escherichia coli* K99

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Abstract

Background: Colicins produced by colicinogenic *Escherichia coli* (CEC) are narrow-spectrum antimicrobial agents that are able to kill or prevent closely related strains.

Objectives: The objective of this study was to evaluate the effect of Colicin on the induction of treated mice in prevention of infection caused by *E. coli* K99.

Materials and Methods: The experiment was conducted on 2 mice groups of 2-week-old (30 in each group). All mice were administered streptomycin sulfate prior to treatment to eliminate resident *E. coli*. Group 1 was orally inoculated with phosphate buffer saline (PBS) as control and the second was fed with colicin solution as treated group. Both control and treated groups were challenged by 3 LD_{50} of *E. coli* K99 and followed up for 1 week.

Results: Treated mice did not show severe clinical signs. While diarrhea with different sings of colibacillosis was established in control group, infected mice showed different clinical signs.

Conclusion: The study indicates that the use of colicin and biotherapy instead of antibiotic may be more safe and efficient for control of *E. coli* K99 infection. Treated mice by colicin solution protected *E. coli* K99 colonization and reduced fecal shedding. Investigation on livestock for applying colicin in animal farms is recommended.

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Background

The neonatal calf, pig, lamb, and kid are mostly involved with many diarrheal diseases.[1] Epidemiologic studies have indicated enterotoxigenic *Escherichia coli* (ETEC) K99 as the major cause of neonatal diarrhea occurring in the first four days of life.[2] The disease has been estimated to be responsible for as much as 30% of the economic losses in animal husbandry.[3] The use of antibiotics for diarrheal diseases has been blamed for the emergence of multidrug resistance in food animals and human medicine.[4,5] The high risk of antimicrobial resistance and increasing antibiotic residues in environment have compelled to look for alternative antibiotics.[4,6] The potential use of bacteriocin-producing bacteria as probiotic and bio-protective agents has recently received increasing attention.[7,8] Colicins are narrow-spectrum bacteriocins produced by colicinogenic *E. coli* (CEC) that are toxic only to bacteria closely related to the producing strain.[9] They are produced by strains of *E. coli* that carry a colicinogenic plasmid, bearing the genetic determinant for colicin synthesis, immunity, and release.[3,5] Surveys have shown that approximately 30% of all *E. coli* strains can produce colicin.[10] Pore-forming colicin, such as colicin V (ColV), binds to its target bacteria, disrupts the ionic gradient of the cell, and then kills it.[5]

Although many studies have investigated the inhibitory effect of colicin against intestinal *E. coli* pathogen,[8,10,11] none of them has explained an experimental in vivo study against *E. coli* K99. However, the study of interaction between pathogenic bacteria and the host needs a simple and cheap animal model.

Objectives

In this research, we investigated the effect of ColV for protection of mice against pathogenic *E. coli* K99 strain.

Patients and Methods

Bacteria

Escherichia coli K99 and CEC isolated from cattle gastrointestinal (GI) tract were previously collected and stored at -70°C.[12,13] Virulence factors and colicin gene of the strains were verified by polymerase chain reaction (PCR).

The strains were revived and inoculated onto the trypticase soy broth (TSB) (HiMedia, India). DNA extraction was carried out by commercial extraction kit (DNA kit Cina-Gene, Iran). Four sets of primers were used for amplification of genes (Table 1).[14,15] A volume of 25 µL PCR mixture was applied for amplification of these genes. PCR mixture included: a 25 µL reaction volume containing 10 × PCR buffer (2.5 µL), 25mM $MgCl_2$, dNTP (10mM), forward and reverse primers (20 pmol), DNA template (100 ng), distilled water (18.5 µL), and Taq DNA polymerase (O.25 µL) (all from Fermentas, Germany). The PCR procedure was performed by Master Gradient Termocycler (Eppendorf, Germany) at 94°C for 3 minutes (one cycle for initial denaturation), 94°C for 30 seconds (denaturation), 56°C for 35 seconds (annealing), and 70°C for 45 seconds (extension), followed by 72°C for 10 minutes (final extension). The stages 2, 3, and 4 were repeated for 25 cycles. The PCR product was stained by etidium bromide, electrophoresed, run on agarose gel, and finally visualized under ultra violet by gel documentation system (Gel Doc, KodaK, USA).

Escherichia coli K99 Preparation

The *E. coli* k99 was grown in Minca broth medium and incubated overnight (O/N) at 37°C. Cells were washed with phosphate buffer saline (PBS) (Kimiateb Co., Iran) by centrifugation at 3000×g for 4 minutes and the number of bacteria was adjusted to 1×10^8 CFU/mL.

Colicin Solution Preparation

The method of Pugsley and Oudega,[16] with some modifications, was used to prepare colicin solution. Briefly, CEC strains were grown in Luria-Bertani (LB) (HiMedia, India) broth and incubated O/N at 37°C. Five milliliters of the O/N culture was inoculated into 50 mL of fresh LB broth and incubated at 37°C for 3 to 4 hours with shaking (160 rpm). When the optical density at 600 nm reached 0.4, mitomycin C solution (250 µg/mL) was added to attain a volume of 500 ng/mL at final concentration (mitomycin induces Colicin production). After 1 hour additional incubation, the broth was disrupted by sonication (UP200H; Hielscher Co. Germany), then centrifuged, and the supernatant was filtrated through low protein-binding 0.22-µm-pore-size membrane filters (Spritzenfilter, Denmark) and stored at -80°C as colicin solution. The production of colicin was proved in previous experiment, before we examine the antibacterial effect of some colicinogenic *E. coli* by Spot method.

Mice Treatment

The experiment was conducted on 2 mice groups of 2-week-old (30 in each group). Prior to bacterial inoculation, all mice were administered 10 mg/L streptomycin sulfate (Jaber-E-BneHayyan Pharmaceutical Mfg. Co., Iran) in their drinking water to eliminate resident *E. coli* facultative bacteria. Antibiotic treated mice were screened for considering fecal *E. coli* by plating on eosin methylene blue (EMB) agar (HiMedia, India) plates. The mice were divided equally into 2 groups. The first group was maintained in a separate cage as control inoculated with oral PBS. All mice in the second group were fed with colicin solution twice a day, with 2-day intervals.

Treated Mice Comparison

Pathogenic *E. coli* K99 was used for challenge. The LD_{50} was estimated according to Yousif et al.[17] Both control and treated groups were challenged by 3 LD_{50} of *E. coli* K99 and followed up for 1 week. Fecal samples from both groups were taken 7 days after challenge. One gram of feces was separately transferred onto 1 mL of PBS. They were serially diluted tenfold (1:10) in PBS (pH 7.2), 200 µL of each dilution was plated onto nutrient agar, and the plates were incubated for 24 hours at 37°C. Colony forming units (CFUs) were monitored per gram feces.

Data Analysis

The results were declared as means ± standard deviation (SD). Analysis of variance (ANOVA) was used to distinguish statistical significant differences ($P<.05$) among various groups. A 2-tailed, paired Student's *t* test was carried out to characterize statistical variation between groups.

Results

Bacterial Detection

PCR was used for determination of the genes encoding the colicins for CEC and virulence factors for *E. coli* K99. Multiplex PCR identified 2 fimbrial (F 5 and F 41) and one *STa* toxin genes of *E. coli* K99 and CEC harbored Col V (Figure 1).

Experimental Challenge

Treated mice group showed mild signs of illness and depression for 2-3 days including loss of appetite and weight, dehydration, ruffled fur, lethargy, and hunched posture. Diarrhea may or may not be seen. They went back to normal condition at the middle of the experiment (4 days after the experiment was started). As noted, *E. coli* k99 was established in GI tract of control group and watery diarrhea was observed with different sings of colibacillosis post challenge. The infected mice could be fatal if not treated within 3-4 days (Figure 2).

Table 1. Oligoneucleotide Primers Used in Multiplex PCR

Gene	Oligonucleotide Primer	Size of Product (bp)
F5	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380
STa	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190
Col V	CAC GCC CTG AAG CAC CAC CA CCG TTT TCC AAG CGG ACC CC	400

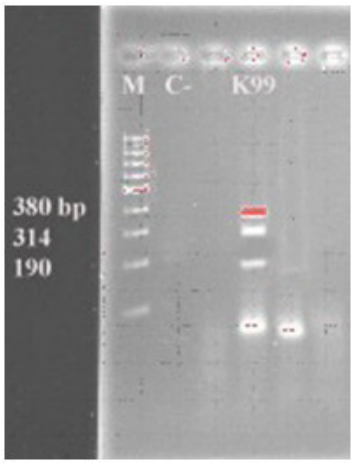

Figure 1. Product of Multiplex PCR on *E. coli* K99 Isolated From Cattle.
Line M: 100 bp ladder, C-: negative control, F41fimbria: 380 bp, K99fimbria: 314, heat stable enterotoxin190 and ColV of colicinogenic *E. coli*: 400 bp.

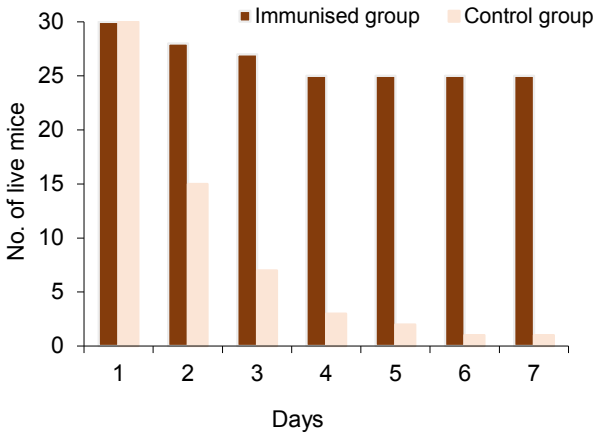

Figure 2. Number of Alive and Dead Mice in Treated and Control Challenge.

Colony Count

The excretion rate of *E. coli* K99 in control and treated mice groups are shown in Table 2. The levels of *E. coli* K99 recovered in the feces of treated group was significantly lower than those in the feces of control group ($P < .05$). However, except for a difference observed at day 1 (less than 1.2 $\log_{10}$ CFU g^{-1}), none of these differences were statistically significant. There were observed significant differences between 2 groups after day 4 (where the differences were more than 1.2 $\log_{10}$ CFU g^{-1}) ($P < .05$). A maximum of 1.262 $\log_{10}$ CFU g^{-1} reduction in *E. coli* K99 was obtained 5 days after colicin solution intake ($P < .05$).

Discussion

We examined the efficacy of ColV in preventing diarrhea caused by *E. coli* K99. Treated mice with colicin solution prevented colonization of *E. coli* K99 in GI tract. An in vitro previous study by Shirazi et al proposed that colicin production by CEC is responsible for the inhibition of *E. coli* k99. Using Spot method, we showed colicin inhibited the growth of *E. coli* K99.[12] The finding of Shirazi et al was comparable with the results of the present study, in which ColV could abrogate lethal activity of *E. coli* K99 in mice.

The use of CEC[9] to colonize in the GI tracts of lamb and kid who suffer from clinical diarrhea is currently considered . Some experiments revealed that colicin removed *E. coli* from GI tract and inhibited the growth of *E. coli* K88, the causative agent of swine diarrhea.[18] Lema et al fed lambs with a mixture of CEC to reduce the incidence of diarrhea, and observed a decrease in pathogen levels.[19] The results indicated that oral administration of *E. coli* K99 after colicin inoculums is more effective than vice versa. This might refer to competitive aspect of CEC. Colicin was primarily replaced in infant GI tract, all receptor binding sites were blocked, and thereafter, *E. coli*

Table 2. Fecal Shedding of *Escherichia coli* K99 by Adult Mice During 7 Days of Experiment

	E. coli K99 Count ($\log_{10}$ CFU g^{-1})																			
	Control Group (n = 30)									Treated Group (n = 30)									diff[3]	*P*
Day	1	2	3	4	5	6	7	8	Ave[1]	1	2	3	4	5	6	7	8	Ave		
1	5.6	5.4	5.5	5.3	5.7	5.2	6.1	5.3	5.513±0.29[a]	4.9	4.4	5	4.5	4.9	4.8	5	4.9	4.8±0.227[a]	0.713	.0439
2	4.3	4.6	4.2	4.5	4.7	4.1	4.4	4	4.35±0.245	3.7	3.4	3.6	3.3	3.5	3.6	3.3	3.4	3.475±0.149	0.875	.0709
3	4.1	4	3.9	3.6	3.7	3.8	3.5	3.6	3.775±0.212	3.9	3.6	3.8	3.1	3.4	3.2	3.1	3	3.388±0.344	0.387	.0467
4	3.3	3.7	3.4	3.3	3.6	3.5	3.8	3.2	3.475±0.212[b]	3	2.6	2.7	2.1	1.9	1.8[e]	2.2	2.3	2.325±0.413[b]	1.42	.0038
5	3.2	3	3.1	2.9	3.3	2.8	2.9	2.9	3.012±0.173[c]	2.2	2.1	2	1.8	1.7	1.5	1.4	1.3	1.75±0.334[c]	1.26	.0041
6	2.7	2.5[l]	2.6	2.4	2.8	2.6	2.4	2.2	2.525±0.191[d]	1.5	1.3	1.7	1.1	1.2	1.2	1	1	1.25±0.245[d]	1.27	.0084
7	2	1	1.3	1.7	1.2	1.4	1.6	1.8	1.5±0.334	1	0.6	0.8	0.7	0.6	0.5	0.4	0.3	0.613±0.223	0.887	.253
D[2]	-	+	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-		

Variables with $P < .05$, in different alphabetical letters, are considered significant.
[1]Average; [2]Diarrhea; [3]Differences (diff) between the mean of control group and treated group in each day.

K99 attachment was inhibited.[9]

The differences between control and test groups in fecal shedding was 1.2 and 1.4 log CFU g^{-1} feces after days 4 and 5, respectively. These differences among other studies were varied, as 1.75, 1.52, 1.6, and 1.8 $\log_{10}$ CFU g^{-1} feces per days 16, 18, 21, and 18, respectively.[10] The mechanism of action of CEC in the inhibition of *E. coli* K99 was not fully understood. It seems that the competitive inhibition of pathogenic bacteria from adhering to respective host receptors, production of anti-bacterial substances such as colicin, and probiotic diversity should be the inhibitory mechanisms of CEC in deterrence of *E. coli* K99 colonization.[20] The rate of the colicin solution that reached the GI tract may have been the ascertaining factor in the appearance of diarrhea.[12]

Colicin significantly decreased the fecal shedding level of the *E. coli* K99 at early hours post challenge. Not all *E. coli* K99 would be killed by colicin solution; in fact, fewer of the *E. coli* K99 was able to cause inflammatory response and produce diarrhea in the treated mice group. Fairbrother et al[21] and Gyles[22] verified this may be due to the low expression of interleukin-1β (IL-1β) in the GI tract tissue treated with colicin solution. During the inflammatory response, IL-1β is firstly secreted by macrophages and active lymphocytes. IL-1β release has been associated with *E. coli* K99 toxin production. Girard et al[23] demonstrated in high dose of colicin intake, the concentration of IL-1β in GI tract was significantly decreased. They also indicated low-dose colicin solution increased IL-1β levels to over six-fold, higher than those in the control group.

In conclusion, this result indicated the potential use of CEC to protect colonization of *E. coli* K99 in GI tract of murine model and reduce fecal shedding and therefore prevent diarrhea. Colicin and biotherapy are good alternative antibacterial agents instead of antibiotic. Investigation on livestock for applying colicin in animal farms is recommended.

Authors' Contributions

FG: preparing the manuscript and laboratory practice. YT: Study design, English editing of the manuscript and Literature review.

Conflict of Interest Disclosures

None.

Ethical Appproval

The institutional review board approved the study.

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