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Enhanced Neomycin Antibiotic Properties Through Proteolytic Enzyme in *Escherichia coli* Induced Infection in Broiler Chicks

Muhammad Shahkar Uzair[1], Muhammad Mushtaq[1], Muhammad Shuaib[1]*, Saqib Nawaz[2], Mehboob Ali[2], Faiz ur Rehman[2], Abubakar Sufyan[3], Usman Zeb[4], Muhammad Ayaz[5] and Muhammad Aamir Khan[5]

[1]*Department of Poultry Science, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan.*
[2]*College of Veterinary Science, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan.*
[3]*Department of Livestock and Poultry Production, Bahauddin Zakariya University, Multan, Pakistan.*
[4]*Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan.*
[5]*Directorate General (Research) Livestock and Dairy Development Department, Khyber Pakhtunkhwa, Peshawar.*

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Serratiopeptidase, Histopathology, Mortality, Neomycin, *In-vitro*

ABSTRACT

The study aimed to explore the beneficial aspect of serratiopeptidase (proteolytic enzyme) combined with low profile antibiotic (Neomycin) in chickens against the *E. coli* infection at the finisher stage. Both in-vitro and in-vivo studies were carried out to evaluate the antimicrobial activities and potency of this combination. A total of one hundred and eighty day-old chicks were randomly allotted to 6 groups i.e. G1 (negative control), G2 (positive control), G3 (standard antibiotic neomycin only), SN-1 (Serratiopeptidase @40g/L+Neomycin@10mg/L), SN-2 (Serratiopeptidase @50mg/L+ Neomycin @20mg/L), and SN-3 (Serratiopeptidase @ 60mg/L+ Neomycin @30mg/L). Each group consisted of thirty birds with 5 replicates (6 birds/ replicate). On day 21, fresh inocula of *E. coli* ($1x10^9$*cfu*) were orally administered to all the groups except negative control. The results revealed that birds in SN-3 group showed significantly larger zone of inhibition, increased feed intake, weight gain and improved feed conversion ratio compared to standard and control groups. Moreover, reduced ($p<0.05$) mortality and morbidity index was found in SN-3 group among all the treated groups. It was concluded, that use of serratiopeptidase combination with neomycin has better impact on broiler chickens to enhance the antimicrobial effect of Neomycin against *E. coli* infection and to enhance production parameters.

INTRODUCTION

Serratiopeptidase (Serratia E-15 protease) is a proteolytic enzyme prepared from plants materials through *Enterobacterium serratia* sp. E-15 found in the intestine of *Bombyx mori* (silkworm). It has a higher anti-inflammatory effect as compared to other proteolytic enzymes and can easily and safely digest non-living tissues which are obtained as a side-product of healing process. For instance, it dissolves scar tissue, arterial plaque, cysts, blood clots and fibrosis. In sinusitis it also acts as mucolytic agent (Robert, 2009). Serratiopeptidase is more active in lungs infections as it removes dead scar tissues and mucous that leads to natural healing of the body tissue through replacement of dead tissues with healthy ones that make possible lung functions normal. It has anti carcinogenic effect as well as it dissolve the outer surfaces like fibroblast breasts which help the body immune system to work against these cancerous substances in the body (Robert, 2009).

Escherichia coli are the naturally occurring microbial flora in poultry and humans gut that cause gastrointestinal

* Corresponding author: shoaibwzr@gmail.com
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diseases. Most of *E. coli* strains are not pathogenic while some pathogenic strains are responsible for causing lesions in immune-compromised hosts (Akond *et al*., 2009). *E. coli* are associated through more severe ailments and occasionally by life threatening infections such as meningitis, endocarditis, urinary tract infection (UTI), septicemia, and epidemic diarrhea in adults and children (Akond *et al*., 2009). Moreover, in poultry it causes yolk sac infection, omphalitis, cellulitis, swollen head syndrome, coligranuloma, and colibacillosis (Gross, 1994).

Neomycin is an aminoglycoside, which is comprised of two or more amino sugars linked by glycosidic bond (Fischer *et al*., 2006). It is naturally produced by the bacterium *Streptomyces fradiae* (Nobel Foundation, 2008). Its Production needs particular nutrient environments in either stationary or submerged aerobic conditions. The compound is then secluded and filtered from the bacterium. Individual effects of these antibiotic and seratopeptidase have been studied in animal model. It is hypothesized in this study that neomycin and seratopeptidase may have synergestic effect against *E. coli* infection. Based on this hypothesis, the present study was planned to assess the efficacy of Seratiopeptidase with Neomycin against *E. coli in-vitro*. It was also the objective of this study to evaluate the efficacy of seratiopeptidases with neomycin on overall performance of broiler chicks.

MATERIALS AND METHODS

Birds husbandry

This study was conducted in the Department of Poultry Science, the University of Agriculture Peshawar, Pakistan. A total of 180 days old chicks were purchased from the local market. They were randomly allocated to six groups including G1 (negative control) means no infection and no treatment, G2 (positive control) means only infection and no treatment, G3 (standard) means infection treated with standard antibiotic Neomycin @ 30mg/L, SN-1 (Serratiopeptidase @40g/L+ Neomycin@10mg/L), SN-2 (Serratiopeptidase @50mg/L+ Neomycin @20mg/L), and SN-3 (Serratiopeptidase @ 60mg/L+ Neomycin @30mg/L). Each group was comprised of 5 replicate (6 birds per replicate). The birds were reared under standard managmental condition for 35 days. Feed was presented *ad libitum* and birds had free access to water. The birds were vaccinated against Newcastle disease, infectious bursal disease and infectious bronchitis.

In-vitro *antimicrobial effect of serratiopeptidase with neomycin*

Liver tissue was collected from *E. coli* infected bird obtained from Poultry Research Unit of the Department of Poultry Science, University of Agriculture, Peshawar, enriched in nutrient broth and incubated at 37°C for 24 h. After incubation the broth was checked for growth and compared with negative control. The inoculum from optimized growth of *E. coli* was taken from nutrient broth and streaked over nutrient agar plates, incubated at 37°C for 24 h for colony morphology. After incubation, the plate was observed for typical *E. coli* colonies. A single colony was picked and subcultured in fresh media followed by confirmation through gram staining. Each of neomycin (30 µg/Disc), serratiopiptidase and neomycin combination at the rate of 1 µl +10 µg, 2 µl +20 µg and 3 µl +30 µg were placed on ager plate at recommended distance (24mm from each other and 12mm from plate edges). All the plates were properly labelled, sealed with parafilm, and incubated at 37 °C for 48 h. Zones of inhibition against the *E. coli* were recorded after 48 h. DMSO (dimethyl sulfoxide) 10% was poured on discs taken as negative control (Buwa and Staden, 2006).

In-vivo *antimicrobial activities of serratiopeptidase with neomycin*

Chicks were placed in metabolic cages 2 weeks before inoculation with *E. coli.* Concentrated inocula (1.0 x 10^9cfu/ml) were prepared in order to induce *E. coli* infection in treated birds (Griffin, 1995). Briefly, *E. coli* on nutrient agar plates were shifted to 10 ml sterilized peptone water test tube and incubated at 37°C for 18-24 h to activate the culture. The tested organism were transferred twice in nutrient broth, and then incubated again for 18-24 h at 37°C. The cell concentration of 1.0 x 10^7cfu /ml was achieved by diluting the active culture serially in nutrient broth (Eman and Hoda, 2008).

Inoculation of E. coli

At day 21 of the experimental trail, fresh and enriched *E. coli* inocula (1.0 x 10^9 CFU/ml) was administered orally through drinking water to all treatment groups according to standard protocol developed by Eman and Hoda (2008). Simultaneously, serratiopeptidase and neomycin were given at the dose rate of 40mg/L+10mg/L, 50mg/L+20mg/L, and 60mg/L+30mg/L through drinking water to groups SN-1, SN-2 and SN-3, respectively. During experiment chicks were assessed weekly for their feed intake, weight gain, morbidity, and mortality.

Statistical analysis

The data were analyzed through statistical packages scientific analysis system (SAS) by using complete randomized design (CRD) according to standard procedure developed by Steel and Torrie (1981). Level of significance $P<0.05$ was considered for variables and

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clinical signs were categorized as mild (listlessness, loss of appetite) labeled as +, moderate (loss of appetite, listlessness, cough, diarrhea) labeled as ++, and severe (listlessness, loss of appetite, cough, diarrhea, labored breathing, yellow droppings, soiled vent) labeled as +++. For Pairwise comparison we performed least significant difference test (LSD).

RESULTS

In the current experiment, significant effects of antimicrobial activity of neomycin and serratiopeptidase against *E. coli* was observed in *in-vitro* test. Highest (22.33 mm) zone of inhibition was recorded at SN-3 treatment (60mg+30mg) as compared to G1 and other treatments while lowest zone of inhibition was observed in G3 group as shown in Figure I. Results regarding the feed intake have been presented in Table I. Post-infection feed intake decreased ($p<0.05$) in G2 group compared to the other treatments groups while higher ($p<0.05$) feed intake was recorded in G1 and SN-3 groups as compared to other groups. Similarly, post infection weight gain was reduced ($p<0.05$) in G2 group of the birds that was not treated with serratiopeptidase and neomycin, followed by the standard group. While the highest ($p<0.05$) weight gain was recorded in the SN-3 group among all groups (Table II). Infected birds of G2 and G3 showed poor FCR compared to G1 group while improved ($p<0.05$) FCR was observed in all treated group compared to G2 and G3. However, numerically best FCR was noted in SN-3 groups among treated groups as shown in the Table III. Results regarding mortality of broiler chicks during the induced pathogenic infection have been shown in the Table IV. The highest mortality was recorded in G2 group, G3 and SN-I groups as compared to other treatment groups. Decreased ($p<0.05$) mortality was observed in SN-3 group among G2, G3, SN-1, SN-2 groups. Morbidity index indicated that the birds in groups of positive control, standard and SN-I showed the highest disease signs while, birds in G1, SN-2 and SN-3 groups showed improved health status.

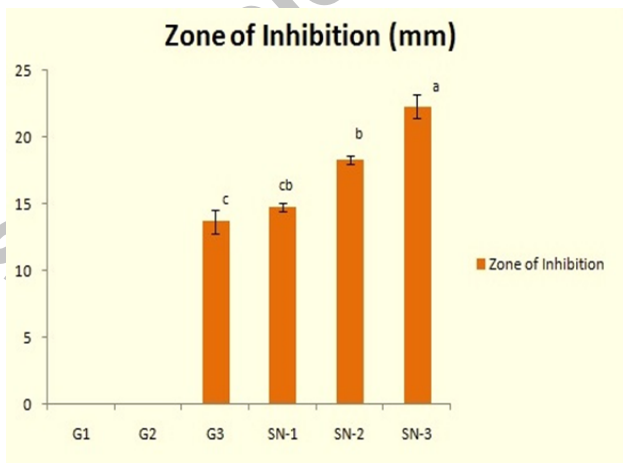

Fig. 1. Comparison of zone of inhibition of seratopeptidase and neomycin against *E. coli* among treated groups.
G1 (negative control), G2 (positive control), G3 (standard antibiotic Neomycin), SN-1 (Serratiopeptidase @40g/L+Neomycin@10mg/L), SN-2 (Serratiopeptidase @50mg/L+ Neomycin @20mg/L), and SN-3 (Serratiopeptidase @ 60mg/L+ Neomycin @30mg/L).

Table I. Effect of serratiopeptidase and neomycin on feed intake (g) in broiler chicks (Mean±SE).

Groups	Week-1	Week-2	Week-3	Week-4	Week-5	Overall
G1	118.3±2.90	299.6±9.06	480.3±8.37	781.6±16.4^{a}	1002±11.0^{a}	2682±47.1^{a}
G2	118±1.52	303.3±5.20	475±12.5	621.6±24.5^{c}	844.6±29.2^{dc}	2362.6±68.6^{d}
G3	116±2.30	292±6.42	466.6±6.76	705±6.80^{b}	893.3±11.6^{bc}	2473±19.0^{bc}
SN-1	118±1.52	297±4.72	472±6.80	701±3.21^{b}	813.6±40.9^{d}	2401.6±38.3^{cd}
SN-2	117.6±2.02	302±3.78	476.3±4.66	735±8.18^{b}	933±19.3^{ba}	2564±29.4^{b}
SN-3	119.6±2.60	298.6±6.74	481±4.93	774.6±15.1^{a}	1009.3±7.62^{a}	2683.3±11.2^{a}
P-value	0.784	0.378	0.788	<0.010	<0.010	<0.010

Means having different superscripts in columns are significantly different at $p<0.05$. G1, negative control; G2, positive control; G3, standard antibiotic; SN-1, Serratiopeptidase @40g/L+Neomycin@10mg/L; SN-2, Serratiopeptidase @50mg/L+ Neomycin @20mg/L; and SN-3, Serratiopeptidase @ 60mg/L+ Neomycin @30mg/L.

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Table II. Effect of serratiopeptidase and neomycin on weekly weight gain (g) in broiler chicks (Mean±SE).

Groups	Week-1	Week-2	Week-3	Week-4	Week-5	Overall
G1	108.3±1.20	215.3±2.02	303±5.85	385.6±6.00^{a}	457.6± 11.3^{b}	1470±25.8^{a}
G2	108.3±1.85	219±0.57	303±4.72	241± 5.56^{d}	307.6± 6.69^{e}	1179±13.00^{e}
G3	108.3±1.20	218.6±1.45	302±6.24	248.6±14.49^{d}	330.3±5.33^{d}	1208±21.0^{d}
SN-1	109.6±2.90	216.6±3.84	303±3.60	330± 5.50^{c}	399.3± 2.84^{c}	1358.6±12.1^{c}
SN-2	109.3±2.02	215.3±2.18	302±1.52	354.3±2.60^{b}	409.6± 5.45^{c}	1390.6±6.11^{b}
SN-3	108±0.57	217.6±2.72	303.6±3.92	375±5.77^{a}	475.3±6.83^{a}	1479.6±12.4^{a}
P-value	0.980	0.785	0.998	<0.010	<0.010	<0.010

Means having different superscripts in columns are significantly different at p<0.05. See Table I for details of group.

Table III. Effect of serratiopeptidase and neomycin on weekly feed conversion ratio (FCR) in broiler chicks (Mean±SE).

Groups	Week-1	Week-2	Week-3	Week-4	Week-5	Overall
G1	1.08±0.01	1.38±0.02	1.58±0.00	2.02±0.01^{c}	2.18±0.03^{cb}	1.81±0.00^{b}
G2	1.08±0.02	1.38±0.02	1.56±0.02	2.57±0.06^{b}	2.74±0.09^{a}	2.00±0.03^{a}
G3	1.06±0.02	1.33±0.02	1.54±0.05	2.85±0.14^{a}	2.70±0.06^{a}	2.04±0.02^{a}
SN-1	1.07± 0.02	1.36±0.01	1.55±0.00	2.12±0.03^{c}	2.03±0.10^{c}	1.96±0.03^{b}
SN-2	1.07±0.03	1.39±0.03	1.57±0.02	2.07±0.02^{c}	2.27±0.02^{b}	1.84±0.02^{b}
SN-3	1.10±0.02	1.36±0.01	1.58±0.03	2.06±0.02^{c}	2.11±0.02^{cb}	1.81±0.01^{b}
P-value	0.742	0.246	0.923	<0.010	<0.010	<0.010

Means having different superscripts in columns are significantly different at p<0.05. See Tables I and II for details of groups.

Table IV. Effect of serratiopeptidase and neomycin on mortality (%) and morbidity index in broiler chicks (Means±SE).

Groups	Mortality (%)	Morbidity index†
G1	00±00^{b}	+
G2	1.00±0.57^{a}	+++
G3	1.00±00^{a}	++
SN-1	1.00±00^{a}	++
SN-2	0.66± 0.33^{ba}	+
SN-3	00±00^{a}	+
P- value	0.053	*****

†Morbidity index: Mild (+), Moderate (++), Severe (+++). For statistical details and details of groups, see Tables I, II and III.

DISCUSSION

In the present study single or combined effect of neomycin and serratiopeptidase was assessed against *E. coli* infection in broiler chicken in vitro as well as *in vivo*. The present results revealed that using neomycin with serratiopeptidase in broiler chicken synergistically improved the antibacterial effect of Neomycin against *E. coli* infection. Treatment @ 60mg+30mg in SN-3 group significantly increased the zone of inhibition than control and other treatment groups. The best results in the aforementioned group of treatment might be due to the activity of serratiopeptidase helping in reducing the bio-film layer of bacteria, also reducing the fluid in the tissues. These results were in similar with the results documented by Sabin *et al*. (2014), and Selan *et al*. (2015). They reported that combination of enzyme with antibiotics showed maximum zone of inhibition against different culture of bacteria as compared to individual effect of antibiotics. The feed intake in experimental birds showed significant variation after *E. coli* induction. Feed intake post infection showed significant decrease in G2 group (positive control) as compared to other treatments groups. However, significantly higher feed intake was recorded in control and SN-3 groups than all other groups. In previous studies, Balevi *et al*. (2000) reported that addition of enzyme does not result in any improvement in feed intake of broiler chicks which is contrary to our study findings. While, Fathi *et al*. (2016) depicted that addition of enzyme had significant effect on feed intake of broiler chicks due to enhancement of enzymatic activity and growth performance. Post infection weight gain was significantly

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reduced in G2 group followed by the G3 group. Highest weight gain was recorded in SN-3 group among other treated groups that was similar to G1 group. Previous findings of Islam *et al*. (2004) and Youssef *et al*. (2014) also supported our findings regarding body weight gain of broiler chicken. The improved weight gain might be linked to mitigation of infection, enhancing the metabolism and feed intake that resulted positive impact. Pre-infection of weekly FCR showed non-significant alteration while post-infection at week 4 and onwards showed significant variation. Positive control (G2 group) and standard (G3 group) showed significantly poor FCR post infection than rest of the groups. Control group and other treatment groups (SN-1, SN-2 and SN-3) showed significantly best FCR. These results were consistent with results documented by Youssef *et al*. (2014) and Mahmoud *et al*. (2017). They reported that better FCR in the treated groups might be due to improved digestive enzyme efficiency. Similarly, Swain *et al*. (1996) also reported similar findings which were in close agreement with current study results. The highest mortality percentage was recorded in G2, G3, and SN-1 group as compared to other treatment groups. While, the morbidity index indicated that the birds in groups of G2, G3, SN-1 showed more disease signs as compared to birds in G1, SN-2 and SN-3 that showed improved health status at post infection phase. This might be due to synergistic effect of seratopeptidase enzyme with neomycin in combating infection. Similar results were also documented by Griggs and Jacob (2005), Jalaludeen *et al*. (2005), and Shawkat *et al*. (2015) as they found decreased morbidity and mortality in chicks after enzyme addition. This effect might be ascribed to improvement in digestive enzyme activity and also producing inhibiting compounds after immune response.

CONCLUSIONS

It is concluded from the results of the current study, that seratiopeptidase combination with neomycin @ 60mg/L and 30mg/L in broilers is highly effective in obtaining production parameters, lowers mortality rate and improving morbidity index and a positive impact on antibacterial activity of neomycin *in-vitro*.

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IRB approval

The study was approved by the Board of Study (No.245/PS/AUP, dated July 27, 2020), conducted at the Department of Poultry Science, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar, KP, Pakistan.

Ethical statement

The experimental procedures used in the study were according to the guidelines of the Ethical Review Committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar. Proper approval was taken by the aforementioned authority before start of experimental trial

Statement of conflict of interest

The authors have declared no conflict of interest.

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