Studies on Performance, Immunity, and Safety of Broilers Vaccinated with Killed H9N2 Vaccine and Supplemented with Essential Oils of Mentofin® in Drinking Water

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ABSTRACT

The aim of this research is to evaluate the effect of intermittent administration of essential oil of Mentofin® on immunity, blood parameters, and growth performance of unvaccinated and vaccinated broilers, administered killed H_9N_2 vaccine followed by controlled challenge with homologous strain of the virus,. Two hundred and forty SPF day-old broiler chicks (Ross 308) were subjected to a completely randomized design, by allocating them to five treatments (A-E), with four replicates/treatment, and 12 birds/replicate. Birds in treatments D and E were vaccinated subcutaneously at seven days of age. Mentofin® was administered intermittently in drinking water to broilers in treatments C and D. An intranasal challenge with H_9N_2 was administered at 28 days of age to birds in treatments B, C, D and E. Results showed that birds in treatment D, vaccinated and treated with Mentofin® and challenged, had the lowest heterophil count and lowest Heterophil/lymphocyte ratio among all challenged birds of the other treatments. In addition, birds in treatment D, compared to all challenged birds, had the highest HI titer associated with lowest enzyme blood profile. Moreover, birds of treatment D had the highest live body weight and lowest feed conversion at 42 days of age compared to birds of all other treatments. In conclusion, the essential oil of Mentofin® in birds of treatment D proved its immunopotentiating effect, with lowest heterophil reaction, thus alleviating injuries of the challenge, as shown with lowest blood enzyme profile, lowest feed conversion ratio and highest live body weight.

INTRODUCTION

Avian influenza is an important poultry disease with the potential to cause major epidemics in humans resulting in significant economic losses. Avian influenza viruses belong to type A of Orthomyxoviridae family, composed of different hemagglutinins (H_1 to H_{18}) and neuraminidase subtypes (N_1 to N_{11}).[1] The combinations of the H and N proteins resulted in 103 subtypes under type A of the influenza viruses.[2]

Since its isolation from Pakistan in 1999, the virus has caused many outbreaks in the young broilers and laying birds in this country. A documented research revealed 98% homology in the sequence of H_9N_2 isolates from Pakistan with that of Hong Kong isolates that were recovered from children. This signifies the pathogenic potential of avian H_9N_2 isolates in humans too.[3,4]

Avian Influenza H_9N_2 produces no clinical signs in wild birds; however, the infection in domestic poultry results in tissue swelling of the periorbital sinuses, nasal and ocular discharge, and severe respiratory distress. Observed mortality in H_9N_2-infected birds ranged between 20% to 60%.[5,6] The main post-mortem findings are the presence of caseous material in the tracheas, bifurcation of extended secondary bronchi, and microscopic severe necrotizing tracheitis, documented by H & E histopathologic procedure.[7]

The application of immunostimulants in poultry production is becoming essential for improvement of broilers' immunity. Some herbal products have immunopotentiating effect, with unknown mode of action.[8] Mentofin®, a natural essential oil product consisting of 10% eucalyptus oil, 10% menthol, 33% liquid builders, and 47% emulsifier, has safely been used in broiler and layer chicken production for approximately 2 decades.[9] It is used in poultry to reduce *Escherichia coli* (*E. coli*) related lesions, mortality from acute Infectious Bursal Disease (IBD), and to alleviate reactions from Newcastle disease (ND) vaccination.[10] It was shown to have a positive effect on weight gain, associated with improvement of the Feed Conversion Rate (FCR) in broilers. It was able to reduce the morbidity and specific lesions after a controlled challenge with infectious bronchitis virus.[11] Eucalyptus and peppermint oils are known to potentiate both the innate-cell mediated and humoral immune responses

Table 1. *Tabulated experimental design*

Treatments	Vaccination	Mentofin® Administration	Challenge with H_9N_2 Virus
A	No	No	No
B	No	No	Yes
C	No	Yes	Yes
D	Yes	Yes	Yes
E	Yes	No	Yes

in chickens. Actually, the administration of these essential oils has a potent immunomodulatory effect on immune response of birds to vaccines.[12]

The H_9N_2 infection in chickens has been an ongoing problem for many years. The persistence of the disease, despite many control measures by the poultry producers, urged scientists to search for alternative approaches for prevention of this ailment in birds.[13]

The hypothesis of this research is to prove or disprove the claims by the manufacturer's of Mentofin® that the intermittent administration of this material in drinking water will immune-stimulate the vaccine response to killed H_9N_2-Avian Influenza vaccine, and results in better performance of broilers that are subjected to a controlled challenge by the virus.

MATERIAL AND METHODS

Experimental Design

Two hundred and forty SPF day-old broiler chicks (Ross 308) were used in a completely randomized block design in five treatments with four replicate pens/treatment, each containing 12 birds. Birds were placed in experimental shed of the Department of Pathology at University of Veterinary and Animal Sciences in Lahore, Pakistan. Birds were offered feed and water ad libitum. During the trial, the birds were provided daily with 23 h of light and 1 h of darkness. Nutrient requirements were provided evenly to birds in all the five treatments, following periodic scheduling of diets according to the Ross 308 catalogue for Nutrition Specifications (2014), namely starter, (day 0-10), grower (day 11-24), and finisher (day 25-42). The description of the five treatments is shown in Table 1.

The vaccination for birds in treatments D and E was at 7 days old. The vaccine was killed Avian Influenza of subtype H_9N_2 (GALLIMUNE™ Flu H_9, Merial), administered subcutaneously in 0.3ml/chick. Mentofin® (EWABO, Wietmarschen, Germany) was administered intermittently to broilers in treatments C and D, at recommended dose of 0.25ml/L, in drinking water, offered at day 4-6, 15-17, 25-31, and 39-41. The intranasal challenge at d 28 with H_9N_2 was given at $1x10^5$ EID_{50}/0.1 ml/per bird. This challenge was restricted to birds in treatments B, C, D, and E.

Source of Virus

The virus used in this challenge was provided by the Veterinary Research Institute (VRI, Lahore). This virus was propagated in 9-11 day-old embryonated eggs. The Embryo Infected $Dose_{50}$ (EID_{50}) was calculated according to procedure described earlier.14

Serology and Lymphocyte Counts

At d 6, all collected sera were examined by the indirect HI test to ensure that they are serologically negative for Avian Influenza H_9N_2 virus.[15] Three birds were selected randomly from each of the four replicate pens per treatment, at each of the following two ages namely, 35 and 42 day-old. The collected sera were examined for AIV titers, using the HI test.16 The HI antibody titers were transformed to log base 2.

At d 35, just 1 week post challenge with the H_9N_2, three broilers per each of the four

Table 2. *Comparison of mean count of Heterophils, Lymphocytes, and Heterophil to Lymphocyte Ratio (H/L) among the five different treatments*

Treatments	Heterophil Count	Lymphocyte Count	H/L Ratio
A (Negative Control)	35.91 ± 1.06^{a}	49.33 ± 0.84^{c}	0.73 ± 0.02^{a}
B (Challenged*)	72.67 ± 0.47^{d}	30.33 ± 1.01^{a}	2.42 ± 0.08^{d}
C (Mentofin® Treated + Challenged)	60.08 ± 1.02^{c}	41.42 ± 1.11^{b}	1.46 ± 0.05^{c}
D (Mentofin® Treated + Challenged + Vaccinated**)	54.08 ± 1.11^{b}	46.83 ± 0.49^{c}	1.14 ± 0.03^{b}
E (Challenged + Vaccinated)	60.00 ± 0.98^{c}	43.67 ± 1.26^{b}	1.39 ± 0.06^{c}

**Challenged with Avian Influenza virus of subtype H_9N_2*
***Vaccinated with oil based killed vaccine of Avian Influenza H_9N_2*
$^{a-d}$Means ± SD within a column followed by different alphabet superscripts are significantly different ($p<0.05$)

replicate pens were randomly selected for sampling blood from their brachial vein. The blood was collected in EDTA containing tubes. Blood smears were prepared from each sample and stained by May-Greenwald-Giemsa-stain.[17] The heterophil to lymphocyte (H/L) ratio, which was calculated based on screening 100 leukocytes per smear under 100 x magnification of the oil immersion lens times 10 magnification of the ocular lens, resulting in total magnification of 1,000 x.18

Blood Enzymes Profile

Blood was collected from three randomly chosen birds of each replicate/treatment at the ages of 27, 35, and, 42 days old. Sera were separated from coagulated blood samples and preserved at -20°C for assessing the alkaline phosphatase (ALKP), alanine aminotransferase (ALT), and aspartate transaminase (AST) activities, using standard kits (Biosystem S.A, Barcelona, Spain).[19]

Feed Conversion Ratio (FCR)

The feed consumption and live body weights of birds in each replicate pen were measured, and the feed conversion ratio FCR was calculated accordingly at the ages of 14, 28, and 42 days old.20

Statistical Analysis

Mean values of data were calculated and analyzed by One-way ANOVA using computer software SPSS (Statistical Package for Social Sciences) version 23. The Tukey's test was applied to find the significant difference between the groups at $P<0.05$.

RESULTS

Results showed that birds in treatment D (vaccinated, treated with Mentofin® and challenged), had the lowest heterophil count

Table 3. *Mean ± SD of Avian Influenza H9N2-antibody titer among different broiler treatments*

Treatments	HI antibody titer (log2)	
	Day 35	Day 42
A (Negative Control)	$0.0\pm 0.00^{a,1}$	$0.0\pm 0.00^{a,1}$
B (Challenged*)	$4.3\pm 0.21^{b,1}$	$5.8\pm 0.12^{b,2}$
C (Mentofin® Treated + Challenged)	$4.6\pm 0.10^{c,1}$	$6.2\pm 0.08^{c,2}$
D (Mentofin® Treated + Challenged + Vaccinated**)	$6.0\pm 0.15^{e,1}$	$7.0\pm 0.14^{e,2}$
E (Challenged + Vaccinated)	$5.4\pm 0.28^{d,1}$	$6.6\pm 0.12^{d,2}$

**Challenged with Avian Influenza virus of subtype H9N2*
***Vaccinated with oil based killed vaccine of Avian Influenza H9N2*
$^{a-e}$Means ± SD within a column followed by different alphabet superscripts are significantly different ($p<0.05$)
$^{1-2}$Means ± SD in a row, followed by Arabic numerical superscripts are significantly different ($p<0.05$)

Table 4. *Effect of Mentofin® on growth performance*

Parameters	Treatments				
	A	B	C	D	E
Body weight (g/bird)					
Day 14	408.21±2.50[a]	408.85±4.55[a]	409.17±4.11[a]	410.15±4.98[a]	408.71±3.71[a]
Day 28	1106.67±30.60[b]	1107.23±28.56[b]	1129.58±17.44[a]	1130.81±14.92[a]	1106.15±32.22[b]
Day 42	2213.85±36.73[c]	2092.25±24.89[d]	2328.42±46.56[b]	2368.77±43.44[a]	2200.19±11.85[c]
Feeds Intake (g/bird)					
Day 0-14	395.88±4.09[ab]	392.59±7.41[b]	395.18±7.97[b]	398.96±6.85[a]	392.96±4.78[b]
Day 0-28	1385.63±41.41[a]	1398.51±39.86[a]	1384.42±24.81[a]	1387.81±40.50[a]	1386.76±44.96[a]
Day 0-42	3916.12±88.17[b]	4063.51±84.70[a]	3935.52±84.77[b]	3857.78±98.14[c]	4031.86±84.90[a]
Feed/Gain (g/g)					
Day 0-14	0.97±0.01[a]	0.96±0.01[b]	0.97±0.02[a]	0.97±0.01[a]	0.96±0.01[b]
Day 0-28	1.25±0.01[a]	1.26±0.02[a]	1.23±0.01[b]	1.23±0.03[b]	1.25±0.02[a]
Day 0-42	1.77±0.03[d]	1.94±0.03[a]	1.79±0.03[c]	1.74±0.03[e]	1.83±0.04[b]

[a-e]Means ± SD within the same row with different superscripts differ significantly (p<0.05)

and lowest Heterophil/lymphocyte ratio among all challenged birds in the other treatments (Table 2).

Serological Findings

Birds in treatment D, compared to all challenged bird, had the highest mean HI titer at the age of 35 and 42 days (Table 3).

Growth Performance

Birds of treatment D had the highest live body weight and lowest feed conversion at 42 days of age, compared to birds of all other treatments (Table 4).

Serum Biochemical Profile

The blood enzyme profile indicated that birds of treatment D had the lowest level of all quantified enzymes, compared to all chal-

Table 5. *Effect of vaccination, Mentofin® treatment, and challenge on Serum Alanine Aminotransferase (IU/L), Serum Aspartate Aminotransferase (IU/L) and Serum Alkaline Phosphatase (IU/L) levels in differently treated broilers*

Parameters	Treatments				
	A	B	C	D	E
Alanine Aminotransferase					
Day 27	8.42±2.02	8.58±2.78	10.25±2.60	9.58±1.51	10.58±1.93
Day 35	10.00±2.92[a]	14.92±2.11[c]	11.58±3.20[aba]	10.33±2.71[a]	14.42±3.00[bc]
Day 42	10.83± 2.55[a]	15.17±3.49[b]	11.67±1.72[a]	11.33±2.27[a]	13.58±3.26[ab]
Aspartate Aminotransferase					
Day 27	163.58±14.30	170.08±14.83	165.42±18.69	163.83±18.32	167.08±12.08
Day 35	168.67±14.23[a]	284.25±20.26[d]	223.08±15.32[c]	189.25±16.96[b]	240.08±19.01[c]
Day 42	166.08±17.15[a]	292.42±29.77[d]	207.17±13.65[b]	177.17±14.41[a]	235.83±8.62[c]
Alkaline Phosphatase					
Day 27	182.83±44.02	186.33±64.87	184.75±43.21	180.58±48.42	185.67±60.64
Day 35	184.33±53.49	258.08±60.64	221.75±71.42	212.42±69.86	238.33±70.33
Day 42	188.25±55.13[a]	274.17±70.34[b]	216.50±61.68[ab]	203.67±62.04[ab]	240.83±95.75[b]

[a-d]Means ± SD within the same row with different superscripts differ significantly (p<0.05)

lenged birds in the other treatments, at the age of 35 and 42 days (Table 5).

DISCUSSION

Mentofin® has a favorable effect on heterophil to lymphocyte (H/L) ratio. It reduced the H/L ratio in broilers in treatment D that were vaccinated and challenged, to a lower level compared to any challenged birds in other groups. These results are in agreement with the findings of previous work,[21] in which eucalyptus supplementation in feed of egg laying hens reduced the H/L ratio significantly. It is documented that stress in birds results in an increase in heterophil associated with a decrease in lymphocyte counts.[22] Moreover, lymphopenia could be due to an infection caused by viruses or other stress-induced factors.[23] This suggests that Mentofin® could alleviate the stress from AIV subtype H_9N_2 in challenged broilers, or acts as an antiviral substance,[24] reducing the pathogenicity of this pathogen and indirectly the heterophil multiplication.

The HI titer specific to AIV subtype H_9N_2 in Mentofin® treated, vaccinated, and challenged broilers of treatment D showed highest antibody titer compared to all challenged broilers deprived of Mentofin® treatment. Previous documentation showed that Mentofin® ameliorates the pathological lesions caused by E.coli along with reduction in mortality rate in clinical IBDV condition in broilers vaccinated with live ND vaccine.[10] Other workers showed that Mentofin® was helpful in improving the acquired humoral immune response against IBDV and NDV in immunosuppressed broilers.[25] Actually, the essential oils of eucalyptus and peppermint showed potent immunomodulatory effect by increasing the humoral antibody titer specific to the hemagglutinin of NDV.[8,12] Moreover, an in vitro study showed the loss of viability of AIV and NDV following a short time contact with Mentofin®, confirmed by their inability to propagate in embryonated chicken eggs compared to their efficient propagation when these viruses are deprived of contact with the Mentofin®.[24]

Birds of treatment D showed highest live body weight at 28 and 42 days of age and lowest feed conversion ratio (FCR) at 42 days of age compared to birds of all other treatments. This data indicates that Mentofin® could be involved in elevating the efficiency in utilization of nutrients. These results are in agreement with previous work,10 in which birds offered Mentofin® were able to reduce the broilers' FCR and to increase their body weight gain, an indicator of potential effect in growth promotion. In another study,[25] an absence of significant difference in weight gain between birds receiving the Mentofin® and the other Mentofin®-deprived birds was reported, but the FCR was apparently improved by the essential oil blend. Actually, the active ingredients in the essential oil blend of Mentofin® were able in Mycoplasma gallisepticum and H_9N_2-combined challenge of broilers to sustain the integrity of the tracheal cilia, prevent mucosal hypertrophy and degeneration of goblet cells, and reduce the infiltration by heterophils,[26] and the mucus formation in air passages, resulting in a decrease in rales frequency; an alleviation of these respiratory tract injuries could be behind the improved FCR and the better live body weight by the essential oil blend.[11]

Mentofin® was able to reduce the Serum biochemical profile of enzymes, reaching to lowest level in birds of Treatment D. Previous studies revealed that eucalyptus and peppermint has ability to decrease the blood physiologic parameters along with hepatoprotective effects.[27,28,29,30] The use of eucalyptus oil reduces the liver enzymatic level in laying Japanese quail as compared to control group.[31] High free radical scavenging capacity of peppermint oil might be helpful in 50% reduction of radical generator 2, 2-diphenyl-1-picrylhydrazyl (DPPH) resulting in increased ALKP level, with absence of an increase in ALT and AST level in rats.[32] In contrast, the peppermint had an impact on dynamics of ALT and AST levels in fattening Sanjabi lambs.[33] In rats, Carbon tetrachloride induced hepatotoxic effects with significant increase in ALT,

AST, ALKP, and GGT levels. Feeding of CCL4 treated rats with peppermint was able to reduce the toxic effect of CCL4, which was associated with significant decrease in ALT, AST, and ALKP values ($p<0.05$).[34] Peppermint had better liver functionality in homeopathy of liver enzymes (eg, ALT and AST), sustaining them within the normal ranges, and with documented effect on growth promotion.[35] The ALT level in serum is commonly used as specific indicator of hepatocellular degeneration as compared to AST and ALKP levels.[36] The return to normal levels of serum transaminases is an indication of healing of hepatocytes after injury.[37]

The results obtained from birds in the treatment D group showed the safety of the Mentofin® in helping the reduction in levels of serum ALT and AST, in spite of the challenge with live strain of H_9N_2. This data is in agreement with a previous documented work.[38] However, it does not agree with other workers who found an elevation in ALKP level after the use of herbal products.[39]

CONCLUSION

In conclusion, the essential oil of Mentofin® in treatment D proved its immunopotentiating effect, with the lowest heterophil reaction, thus alleviating injuries of the challenge, as shown with the lowest blood enzyme profile, lowest feed conversion ratio, and highest live body weight.

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