2020

Association of Chicken Mx1 Polymorphisms with Susceptibility in Chicken Embryos Challenged with Virulent Newcastle Disease Virus

Mpenda, Fulgence

Science Alert

https://scialert.net/abstract/?doi=ajas.2020.9.15

OPEN ACCESS Asian Journal of Animal Sciences

ISSN 1819-1878
DOI: 10.3923/ajas.2020.9.15

Research Article
Association of Chicken Mx1 Polymorphisms with Susceptibility in Chicken Embryos Challenged with Virulent Newcastle Disease Virus

[1]Fulgence Ntangere Mpenda, [2]Sylvester Leonard Lyantagaye and [1]Joram Buza

[1]Department of Global Health, School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Tengeru, Arusha, Tanzania
[2]Department of Molecular Biology and Biotechnology, College of Natural and Applied Sciences, University of Dar es Salaam, Dar es Salaam, Tanzania

Abstract

Background and Objective: Genetic selection of chicken genotypes that are less susceptible to Newcastle disease virus (NDV) is a promising option. The present study aimed to investigate polymorphism of chicken Mx1 gene G2032A SNP in different chicken breeds in Tanzania and association with chicken embryos susceptibility to virulent NDV. **Materials and Methods:** A total of 355 (87 Sasso, 129 Kuroiler and 139 local) 16 days old chicken embryos were infected with 0.1 mL minimum lethal dose (10^3/0.1 mL) of virulent NDV suspension. The time of death was recorded and selective genotyping was deployed where chicken embryos from high (15%) and less (15%) susceptible cohorts were genotyped by PCR-length polymorphism. Two forward and a reverse primers were used to generate 2 different sizes of PCR product of A and G alleles of chicken Mx1 gene G2032A SNP. **Results:** As expected chicken embryos survival was highly variable within a breed. The frequency of allele A was higher than allele G in chicken breeds. Furthermore, using Pearson's X^2 test of independence, it was demonstrated that chicken Mx1 gene G2032A genotypes (AA, AG and GG) were associated ($p<0.05$) with chicken embryos susceptibility to virulent NDV infection. The frequency of genotype AA was higher in less susceptible chicken embryos, whereas, frequency of genotype GG and AG was higher in high susceptible chicken embryos. **Conclusion:** Results from the present study provide valuable information on the potential role of Mx1 gene polymorphism for selection of chickens that are resistant to virulent NDV infection.

Key words: Chicken, genotype and allele frequency, Newcastle disease virus, chicken Mx1 gene

Citation: Fulgence Ntangere Mpenda, Sylvester Leonard Lyantagaye and Joram Buza, 2020. Association of chicken Mx1 polymorphisms with susceptibility in chicken embryos challenged with virulent newcastle disease virus. Asian J. Anim. Sci., 14: 9-15.

Corresponding Author: Fulgence Ntangere Mpenda, Department of Global Health, School of Life Science and Bioengineering, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Tengeru, Arusha, Tanzania Tel: +255769933961

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Newcastle disease (ND) is the principal constraint to chicken production in backyard production systems[1]. The disease is caused by the Newcastle disease virus (NDV), an avian Paramyxovirus serotype-1 (AMPMV-1)[1,2]. The disease has enormous economic impact on the global poultry industry due to losses caused by high morbidity and mortality rates[2-4]. Newcastle disease control and prevention depends on proper vaccination and good bio-security measures[3,5]. However, the disease control and prevention in backyard chickens remains a challenge because of poor adoption of vaccines[6,7] and lack of feasibility of bio-security measures[8]. These limitations prompt the search for alternative control strategies in backyard chicken production systems.

Genotypic individual variations within and between chicken types have been documented[9,10]. Using these variations, chicken lines that are less susceptible to Marek's disease (MD) have been under development for years[9]. Variations in susceptibility to NDV have been reported in inbred and outbred chicken populations[11-13], which provides a promising platform for selection of resistant chicken to NDV.

Using adult chicken to evaluate disease susceptibility is expensive and prone to confounding factors and is less feasible, specifically in resource-poor countries[14-16]. In contrast, chicken embryo under the eggshell environment is less prone to confounding variables and relatively cheap[15] and therefore, an interesting model for evaluating chicken susceptibility to diseases. Association between chicken Mx1 G2032A polymorphism and disease resistance or susceptibility has been demonstrated by challenging chicken embryos (*in ovo*) with less or high pathogenic Influenza virus[17].

However, information on the association between chicken Mx1 G2032A polymorphism and disease resistance or susceptibility by challenging chicken embryos with virulent NDV is lacking and more importantly, the level of chicken Mx1 G2032A polymorphism in local Tanzanian chicken and exotics (kuroiler and Sasso) is not known. The present study aimed to investigate polymorphism of chicken Mx1 gene G2032A SNP in different chicken breeds in Tanzania and association with chicken embryos susceptibility to virulent NDV.

MATERIALS AND METHODS

A total duration of the experiment was 12 months. The study was conducted from January to December, 2018 at Nelson Mandela African Institution of Science and Technology (NM-AIST), Arusha, Tanzania. The experiment started with characterization of virulent NDV field isolate and was followed by embryonated chicken eggs challenge experiment and molecular genotyping of chicken Mx1 gene.

Biological characterization of the virulent NDV field isolate: The virulent NDV was isolated at Sokoine University of Agriculture in Morogoro Tanzania and was kindly provided for the experiment.

Virus propagation, pathogenicity test and titration: The virus was propagated and titrated, as previously described[18-20]. Briefly, 5, 10 days old embryonated chicken eggs were each inoculated with 0.1 mL of virus homogenate into the allantoic cavity and negative control eggs were inoculated with 0.1 mL of phosphate-buffered saline (PBS). Infected embryonated eggs were incubated at 37°C and were first candled 24 h post-infection (pi) for detection of early embryos death, a sign of bacterial contamination. Subsequently, CEs were candled after every 12 h for a total of 96 h. Dead embryos were chilled at 4°C overnight before collection of allantoic fluid for hemagglutination (HA) and hemagglutination inhibition (HI) tests.

Chicken red blood cells (cRBCs) were prepared following the protocol described in the previous study[21]. Briefly, whole blood (3 mL) was drawn from the wing vein using 2.5 mL syringe into a tube containing 3 mL of Alsever's solution[22]. Then, the blood was washed 3 times with PBS. The HA was performed using 0.5% CRBCs in 96-well V bottomed micro-titration plates as previously described[23]. The HI test was conducted using NDV antiserum to confirm the presence of NDV. Positive allantoic fluids were pooled, aliquoted and stored at -80°C.

Then, allantoic fluid was tenfold (10^{-1} to 10^{-9}) serially diluted in sterile PBS. The 0.1 mL of allantoic fluid inoculated in the allantoic cavity of 10 days old embryonated egg and a total of 5 embryos were inoculated with each dilution. The inoculated eggs were treated as described above, with the exception that after 1st candling eggs were examined twice every day for a total of 7 days and embryo time of death was recorded. The minimum lethal dose (MLD), the highest dilution that killed all inoculated embryos, was established. The mean death time (MDT) of embryonated eggs, defined as the average time at which the eggs inoculated with MLD died, was also calculated.

Molecular characterization of the virus

RNA extraction and cDNA synthesis: The viral RNA was extracted by using Kit (QIAGEN) where the manufacturer instructions were adhered. The RNA quality and quantity assessed with the use of NanoDrop 2000 spectrophotometer (Thermo Scientific). The cDNA was synthesized by using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolab, NEB). Random primers supplied by Kit manufacturer (NEB) were used for the cDNA synthesis. The reaction volume was 20 µL, which was performed in 3-step reaction conditions: Incubation at 25°C for 5 min, reverse transcription at 42°C for 1 h and enzyme inactivation at 80°C for 5 min. Negative controls were treated with the same reaction conditions with the exception that nuclease-free water was placed in a reaction volume instead of the viral RNA template.

Polymerase chain reaction (PCR): Molecular characterization of virus isolate was performed using 2 sets of primers (Table 1). The M primer designed to target the Matrix gene for general NDV detection[24], whereas, F primer is designed to identify the virulent NDV strains by targeting the conserved region of 374 base pair (bp) in length, which includes the F protein cleavage site in the NDV genome[25].

The cDNA amplification was conducted by using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) according to the manufacturer instructions. The 20 µL PCR reaction volume in 96-well reaction plate (MicroAmp®Fast, Applied Biosystems) contained the primer final concentration of 0.5 µM and cDNA template final concentration of $\leq$500 ng. The reaction conditions were initial holding denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, primer annealing at 56°C for 30 sec, an extension at 68°C for 1 min and a final extension at 68°C for 5 min. The PCR reaction ran on a QuantStudio 6 Flex real-time (RT) PCR Thermal Cycler (Applied Biosystems). The PCR products were visualized on 1% (w/v) agarose gel in 1x TBE buffer containing ethidium bromide. The amplicons size was compared with the 100bp DNA ladder (NEB). After gel electrophoresis, the bands were visualized under UV light.

Bioinformatics and phylogenetic analysis: The PCR product (~750 bp) of partial F protein gene of the isolate was sent to Inqaba Biotechnology (South Africa) for Sanger's dideoxy chain-termination sequencing method. The forward and reverse raw electropherogram sequences were manually edited and consensus sequence was generated in BioEdit v 5.0.6. Initially, the sequence was translated into protein and an open reading frame was identified using ExPASy translate tool[26] to determine the presence of multiple basic amino acids residues at 112-116 and phenylalanine residue at 117.

Experimental infection of chicken embryos: The study involved local Tanzanian chickens and exotic chickens: Kuroiler[27] and Sasso[28]. A total of 355 chicken embryos (87 Sasso, 129 Kuroiler and 139 local) were tested in 3 experimental replicates. Chickens had the same history of vaccination against NDV. Chicken variability in susceptibility to virulent NDV was evaluated by inoculating 16 days old chicken embryos (CEs) with 0.1 mL (10^3 MLD/0.1 mL) of virus suspension. The CEs were candled after 24 h pi to detect early dead embryos, which may be a sign of bacterial contamination. Subsequent CEs candling was conducted after every 6 h for further 96 h pi to establish CEs death time (DT) differences.

DNA extraction and genotyping of chicken Mx1 gene G2032A SNP: In the present study the selective genotyping strategy was deployed as previously described[29,30]. Therefore chicken embryos from high (15%) and less (15%) susceptible cohorts were selected for genotyping. Genomic DNA was extracted from leg tissues using Quick-DNA Tissue/Insects Kit (Zymo Research) in accordance with manufacturer protocol. The quantity and quality of genomic DNA was measured by using NanoDrop 2000 spectrophotometer (Thermo Scientific) and the integrity of genomic DNA was visualized on 1% (w/v) agarose gel in 1% TBE buffer containing ethidium bromide. A total of 102 samples (40 kuroiler, 38 local chicken and 24 sasso) were suitable for further analysis.

Genotyping of chicken Mx1 gene G2032A SNP was performed by PCR length Polymorphism (PCR-LP) as previously described[17] with minor modification. The primers (+MX1SER: 5'-GCTCTCCTTGTAGGGAGCCAG-3', +MXASN: 5'-TAATAATA ATAACCTCTCCTTGTAGGGAGCGAA-3' and -MX1SERASN: 5'-GTGACTAATTCTGCTGGTCAGTAAC-3') that were previously designed by Wang *et al.*[17], was used for the PCR-LP. The primers amplify a DNA fragment that includes G2032A substitution in the coding region f chicken Mx1 gene.

Table 1: Set of primers used for amplification of partial M and F gene of Newcastle disease virus

Gene	Primers	Sequences (5-3)	Amplicon size (bp)	Annealing temperature (°C)	References
Matrix (M)	4100F/4220R	AGT GATGTGCTC GGA CCT TC CCT GAGGAGAGG CATTTG CTA	121	58	Wise *et al.*[24]
Fusion (F)	4331F/5090R	GAGGTTACCTCYACYAAGCTRGAGA TCATTAACAAAYTGCTGCATCTTCCCWAC	750	58	Kim *et al.*[25]

The PCR conditions were initial denaturation at 94°C for 2 min, which was followed by 40 cycles at 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min. After amplification, the PCR products were loaded on 2.5% (w/v) agarose gel and run in a 0.5% TBE buffer at 80 V for 4 h. Genotyping was possible because of the different size of PCR products generated by forward primers for alleles G (~199 bp) and A (~211 bp).

Ethical statement: The experiment was conducted in compliance with the Guidelines on the Humane Treatment of Laboratory Animals as stipulated in the Tanzania Animal Welfare Act, 2008.

RESULTS

Biological and molecular characterization of the virus isolate: The presence of hemagglutinating agent of NDV was detected in the homogenate of field isolate and the homogenate reacted with monospecific antiserum specific against NDV. Also, MDT of 10 day-old embryonated chicken eggs that were infected with the isolate was 55.5 h with a death time ranged from 36-72 h. The partial Fusion (F) and Matrix (M) gene was amplified further confirming the presence of NDV in the suspension (Fig. 1). Furthermore, through molecular analysis of partial F gene of NDV field isolate DNA sequence, a motif "R-R-Q-K-R-F" suggestive of virulent strain was detected.

Polymorphism of chicken Mx gene G2032A SNP and chicken embryos survival variability: Chicken embryos survival time upon challenge with virulent NDV was highly variable within a breed and the coefficient of variations between breeds was different ($p<0.05$). The MDT and median death time statistics of chicken embryos post challenge with virulent NDV are summarized in Table 2.

A PCR-LP using 2 forward primers (+MX1SER and +MX1ASN) and a reverse primer (-MX1SERASN) produced 2 different sizes of PCR products of A and G allele (Fig. 2). The frequency of an allele A was higher compared to the frequency of an allele G in all chicken breeds (Table 3). The frequency of an allele A was highest in Sasso (0.66) as compared to Kuroiler (0.64) and local chicken (0.64). Also, at the genotype level, the homozygous AA genotype had higher frequency compared to homozygous GG genotype (Table 4).

Pearson's Chi-squared test of independence and Likelihood Ratio tests demonstrated an association ($p<0.05$) between chicken Mx1 gene G2031A genotypes and chicken embryos variation in susceptibility to virulent NDV infection. The allele frequency was associated ($p<0.05$) with the chicken embryos variation in susceptibility to virulent NDV in kuroiler and local Tanzanian chicken embryos (Table 3). Also, the frequency of homozygous AA genotype was higher in less susceptible chickens, whereas the frequency of homozygous GG genotype and heterozygous AG genotype were higher in high susceptible chickens (Table 4).

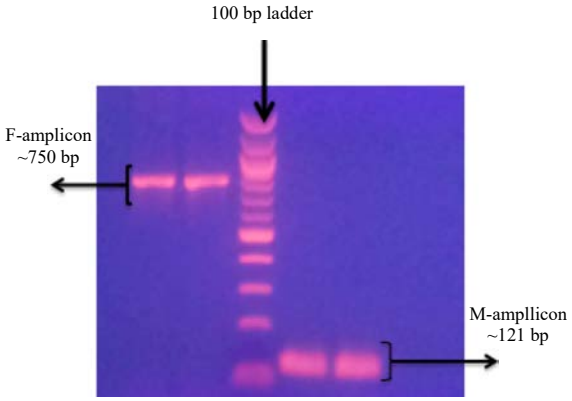

Fig. 1: PCR product sizes of partial F and M gene of NDV observed in the study

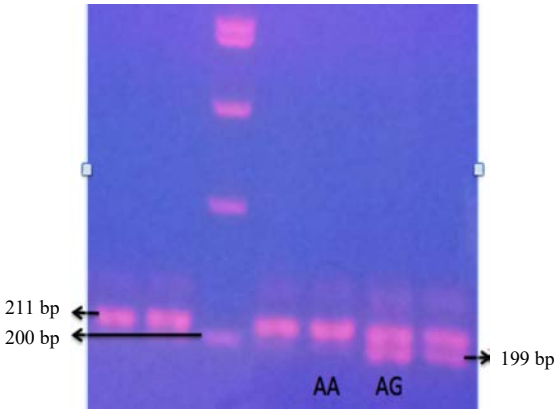

Fig. 2: Polymerase chain reaction length polymorphism (PCR- LP) genotyping of chicken Mx gene G2032A SNP
Example of PCR-LP products separation indicating alleles A (211 bp) and G (199 bp). AA: Homozygous A allele, AG: Heterozygous

Table 2: Statistics of chicken embryos survival time post-challenge with minimum lethal dose of virulent Newcastle disease virus observed in the present study

Chicken types	Mean (h)	SEM	Median (h)*	25th percentiles (h)	75th percentile (h)	IQR (h)
Kuroiler	62.3	1.5	66	54	78	24
Local	60.8	1.1	66	54	66	12
Sasso	76.5	2.1	78	66	90	24

SEM: Standard error of the mean, *Median represent 50th percentiles, IQR: Interquartile range

Table 3: Chicken Mx gene G2032A allele frequencies and association with chicken embryos susceptibility to virulent NDV challenge observed in the present study

		Susceptibility					
Breeds	Alleles	High	Low	χ^2	p-value	LR	p-value
Kuroiler	A	20 (0.50)	31 (0.78)	$\chi^2 = 6.545$		LR = 6,67	
	G	20 (0.50)	9 (0.22)		p = 0.011		p = 0.009
Local	A	19 (0.50)	30 (0.79)	$\chi^2 = 6.951$		LR = 7.105	
	G	19 (0.50)	8 (0.21)		p = 0.008		p = 0.008
Sasso	A	17 (0.71)	15 (0.63)	$\chi^2 = 0.375$		LR = 0.376	
	G	7 (0.29)	9 (0.37)		p = 0.540		p = 0.539

χ^2: Pearson's Chi-squared test of independence (association), LR: Likelihood ratio, p<0.05 considered statistically significant

Table 4: Chicken Mx gene G2032A genotype frequencies and association with chicken embryos susceptibility to virulent NDV challenge observed in the present study

		Susceptibility					
Breeds	Genotypes	High	Low	χ^2	p-value	LR	p-value
Kuroiler	AA	6 (0.30)	11 (0.55)	$\chi^2 = 7.529$		LR = 8.869	
	AG	8 (0.40)	9 (0.45)		p = 0.023		p = 0.007
	GG	6 (0.30)	-				
Local	AA	4 (0.21)	13 (0.68)	$\chi^2 = 8.69$		LR = 9.093	
	AG	11 (0.58)	4 (0.21)		p = 0.013		p = 0.011
	GG	4 (0.21)	2 (0.11)				
Sasso	AA	6 (0.50)	4 (0.34)	$\chi^2 = 0.733$		LR = 0.737	
	AG	5 (0.42)	7 (0.58)		p = 0.693		p = 0.692
	GG	1 (0.08)	1 (0.08)				

χ^2: Pearson's chi-squared test of independence (association), LR: Likelihood ratio, p<0.05 considered statistically significant

DISCUSSION

The present study was aimed to test a null hypothesis that local Tanzanian, Kuroiler and Sasso chicken embryos susceptibility to virulent NDV infection is independent of chicken Mx1 gene G2032A genotypes (AA, AG and GG) or alleles (A and G). Results demonstrated for first time an association between chicken embryos susceptibility to virulent NDV challenge and chicken Mx1 gene G2032A genotypes and alleles. The frequency of genotype AA was higher in less susceptible chicken embryos, whereas, the frequency of genotype GG and AG was higher in highly susceptible chicken embryos.

Characterization of NDV field isolate to confirm virulence of the strain provided a platform to conduct the experiment. The MDT of 10 days old embryonated eggs was 55.5 h, which was in an agreement with MDT range of <60 h when 9-11 days old embryonated chicken eggs are infected with virulent NDV[2]. The biological characterization was confirmed with the use of molecular approach where multiple basic amino acid motif ("112-R-R-Q-K-R-F-117") at F protein cleavage site was detected. The OIE guideline requires characterization of NDV virulence with an intracerebral pathogenicity index (ICPI) of $\geq$0.7 in day-old chicks or molecular determination of the presence of multiple basic amino acids at the F protein cleavage site[18,19]. The presence of multiple basic amino acids at the F0 cleavage site is an essential criterion for confirming the virulence of NDV[18].

Chicken embryos become immunocompetent before hatch and *in ovo* vaccinations and gene expression studies have been harnessing the immune competence of developing chicken embryos[14-16]. In the present study, survival time of chicken embryos post-challenge with virulent NDV was used as a measure of susceptibility. As expected, the death time within a breed was highly variable. The high variability in death time within a breed may be explained by heterogeneity nature of the study populations. The 3 breeds under study (Kuroiler, local Tanzanian chicken and Sasso) were outbred populations and therefore individual genetic differences within a breed are common[31].

The Mx protein is an interferon-induced guanosine triphosphatase, which has been demonstrated to confer resistance to viruses[14-16]. The chicken Mx protein is composed of 705 amino acids, which is encoded by the Mx gene (GeneBank, accession No. Z23168). A single nucleotide polymorphism (SNP) of the Mx protein that affect antiviral activity in chickens has been reported[14,15,17]. The S631N mutation caused by G2032A SNP of Mx cDNA result in elevated antiviral function. An allele A is considered resistant, whereas, an allele G is considered susceptible and likewise

chickens with genotype AA are considered resistant, whereas birds with genotype GG are considered susceptible[32,33]. More importantly, advantageous allele A frequency is reported to be high in chicken populations all over the world suggesting positive selection of an allele[16,17]. Indeed, in the present study, the frequency of allele A was higher compared to the frequency of allele G in all chicken breeds (Table 3). The observed high frequency of allele A is in agreement with the previous reports [34,35]. For example, Indian native chicken, Aseel and Kadaknath had allele A frequency of 0.75 and 0.63, respectively[36]. The highly skewed frequency of A allele may be explained by environmental selection pressure like a persistent exposure to infectious diseases.

Genotypes AA and AG of chicken Mx gene G2032A SNP were higher in less susceptible compared to genotype GG. Pagala *et al.*[35] reported similar findings upon challenging adult chickens with NDV where genotypes AA and AG of Tolaki chickens had higher resistance against NDV infection. The less susceptibility of AA and AG genotypes may be conferred by the presence of resistant A allele, whereas, the high susceptibility of GG genotypes may be due to the presence of susceptible G allele. Chicken Mx gene G2032A polymorphism resulting in a substitution of serine with asparagine at position 631 of the chicken Mx protein[32,33].

Results of the present study demonstrated association between chicken Mx1 gene polymorphism, in particular G2032A mutation with chicken embryos survival variation following challenge with virulent NDN and therefore, could be used in breeding programs designed to develop chicken that are less susceptible to NDV infection.

SIGNIFICANCE STATEMENT

The study has further demonstrated the possible role of chicken Mx1 G2032A polymorphisms in chicken resistance to viral infections. Furthermore, the findings demonstrated the robustness of the selective genotyping approach in complex genetic experiments that are very expensive and therefore, it can be useful in resource-poor countries.

CONCLUSION

Chicken Mx1 gene G2032A polymorphism was associated with local Tanzanian, kuroiler and Sasso chicken embryos susceptibility to virulent NDV infection. The frequency of genotypes AA and AG was higher in the less susceptible cohort as compared to genotypes GG that was higher in the highly susceptible cohort.

ACKNOWLEDGMENTS

We thank the Bill and Melinda Gates Foundation for funding this research (Grant No. OPP1083453) through the Program for Enhancing Health and Productivity of Livestock (PEHPL) and the Nelson Mandela African Institution of Science and Technology in Arusha (NM-AIST), Tanzania for hosting the program.

REFERENCES

1. Alexander, D.J., J.G. Bell and R.G. Alders, 2004. A technology review: Newcastle disease with special emphesis on it's effects on village chickens. FAO Animal Production and Health Paper No. 161, Food and Agriculture Organization of the United Nations, Rome, Italy.
2. Alexander, D.J., 2000. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Tech., 19: 443-462.
3. Alexander, D.J., 2001. Newcastle disease. Br. J. Poult. Sci., 42: 5-22.
4. Pedersen, J.C., D.A. Senne, P.R. Woolcock, H. Kinde and D.J. King *et al.*, 2004. Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002-2003 outbreak in California and other recent outbreaks in North America. J. Clin. Microbiol., 42: 2329-2334.
5. Sharif, A., T. Ahmad, M. Umer, A. Rehman and Z. Hussain, 2014. Prevention and control of Newcastle disease. Int. J. Agric. Innovat. Res., 3: 454-460.
6. Blackie, S., 2014. Village chicken production system in the greater Accra region Ghana. J. Biol. Agric. Healthc., 4: 89-94.
7. Campbell, Z.A., T.L. Marsh, E.A. Mpolya, S.M. Thumbi and G.H. Palmer, 2018. Newcastle disease vaccine adoption by smallholder households in Tanzania: Identifying determinants and barriers. Plos One, Vol. 13, No. 10. 10.1371/journal.pone.0206058.
8. Conan, A., F.L. Goutard, S. Sorn and S. Vong, 2012. Biosecurity measures for backyard poultry in developing countries: A systematic review. BMC Vet. Res., Vol. 8. 10.1186/1746-6148-8-240.
9. Bacon, L.D., H.D. Hunt and H.H. Cheng, 2000. A review of the development of chicken lines to resolve genes determining resistance to diseases. Poult. Sci., 79: 1082-1093.
10. Miller, M.M. and R.L.Taylor Jr., 2016. Brief review of the chicken major histocompatibility complex: The genes, their distribution on chromosome 16 and their contributions to disease resistance. Poult. Sci., 95: 375-392.
11. Msoffe, P.L.M., U.M. Minga, J.E. Olsen, M.G.S. Yongolo, H.R. Juul-Madsen, P.S. Gwakisa and M.M.A. Mtambo, 2001. Phenotypes including immunocompetence in scavenging local chicken ecotypes in Tanzania. Trop. Anim. Health Prod., 33: 341-354.

12. Hassan, M.K., M.A. Afify and M.M. Aly, 2004. Genetic resistance of Egyptian chickens to infectious bursal disease and Newcastle disease. Trop. Anim. Health Prod., 36: 1-9.
13. Lwelamira, J., G.C. Kifaro, P.S. Gwakisa and P.L.M. Msoffe, 2008. Association of LEI0258 microsatellite alleles with antibody response against Newcastle disease virus vaccine and body weight in two Tanzania chicken ecotypes. Afr. J. Biotechnol., 7: 714-720.
14. Gimeno, I.M., N.M. Faiz, A.L. Cortes, T. Barbosa, T. Villalobos and A. Pandiri, 2015. *In ovo* vaccination with Turkey *Herpesvirus hastens* maturation of chicken embryo immune responses in specific-pathogen-free chickens. Avian Dis., 59: 375-383.
15. Schilling, M.A., R. Katani, S. Memari, M. Cavanaugh and J. Buza *et al*., 2018. Transcriptional innate immune response of the developing chicken embryo to newcastle disease virus infection. Front. Genet., Vol. 9. 10.3389/fgene.2018.00061.
16. Alkie, T.N., A. Yitbarek, D.C. Hodgins, R.R. Kulkarni, K.S. Taha-Abdelaziz and S. Sharif, 2019. Development of innate immunity in chicken embryos and newly hatched chicks: A disease control perspective. Avian Pathol., 48: 288-310.
17. Wang, Y., V. Brahmakshatriya, B. Lupiani, S. Reddy and R. Okimoto *et al*., 2012. Associations of chicken Mx1 polymorphism with antiviral responses in avian influenza virus infected embryos and broilers. Poult. Sci., 91: 3019-3024.
18. Aldous, E.W., J.K. Mynn, J. Banks and D.J. Alexander, 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol., 32: 239-257.
19. Farooq, M., U. Saliha, M. Munir and Q.M. Khan, 2014. Biological and genotypic characterization of the Newcastle disease virus isolated from disease outbreaks in commercial poultry farms in Northern Punjab, Pakistan. Virol. Rep., 3: 30-39.
20. Heiden, S., C. Grund, A. Röder, H. Granzow, D. Kühnel, T. C. Mettenleiter and A. Römer-Oberdörfer, 2014. Different regions of the Newcastle disease virus fusion protein modulate pathogenicity. Plos One., Vol. 9, No. 12. 10.1371/journal.pone.0113344.
21. Grimes, S.E., 2002. A basic Laboratory manual for the small-scale production and testing of I-2 Newcastle disease vaccine. RAP Publication, 2002/22. http://www.fao.org/3/a-ac802e.pdf
22. Bukantz, S.C., C.R. Rein and J.F. Kent, 1946. Studies in complement fixation: II. Preservation of sheep's blood in citrate dextrose mixtures (modified Alsever's solution) for use in the complement fixation reaction. J. Lab. Clin. Med., 31: 394-399.
23. Alexander, D.J. and N.J. Chettle, 1977. Procedures for the haemagglutination and the haemagglutination inhibition tests for avian infectious bronchitis virus. Avian Pathol., 6: 9-17.
24. Wise, M.G., D.L. Suarez, B.S. Seal, J.C. Pedersen and D.A. Senne *et al*., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Clin. Microbiol., 42: 329-338.
25. Kim, L.M., D.J. King, D.L. Suarez, C.W. Wong and C.L. Afonso, 2007. Characterization of class I Newcastle disease virus isolates from hong kong live bird markets and detection using real-time reverse transcription-PCR. J. Clin. Microbiol., 45: 1310-1314.
26. Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel and A. Bairoch, 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res., 31: 3784-3788.
27. Fleming, D.S., J.E. Koltes, A.D. Markey, C.J. Schmidt and M.F. Ashwell *et al*., 2016. Genomic analysis of Ugandan and Rwandan chicken ecotypes using a 600 k genotyping array. BMC Genomics, Vol. 17. 10.1186/s12864-016-2711-5
28. Amponsah, R.O., B.B. Kayang and A. Naazie, 2012. Age, Genotype and Sex effects on growth performance of local chickens kept under improved management in Ghana. Trop. Anim. Health Prod., 44: 29-34.
29. Darvasi, A. and M. Soller, 1992. Selective genotyping for determination of linkage between a marker locus and a quantitative trait locus. Theor. Applied Genet., 85: 353-359.
30. Sen, Ś., F. Johannes and K.W. Broman, 2009. Selective genotyping and phenotyping strategies in a complex trait context. Genetics, 181: 1613-1626.
31. Mpenda, F.N., M.A. Schilling, Z. Campbell, E.B. Mngumi and J. Buza, 2019. The genetic diversity of local African chickens: A potential for selection of chickens resistant to viral infections. J. Applied Poult. Res., 28: 1-12.
32. Ko, J.H., H.K. Jin, A. Asano, A. Takada and A. Ninomiya *et al*., 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res., 12: 595-601.
33. Ko, J.H., A. Takada, T. Mitsuhashi, T. Agui and T. Watanabe, 2004. Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631. Anim. Genet., 35: 119-122.
34. Li, X.Y., L.J. Qu, J.F. Yao and N. Yang, 2006. Skewed allele frequencies of an Mx gene mutation with potential resistance to avian influenza virus in different chicken populations. Poult. Sci., 85: 1327-1329.
35. Pagala, M.A., Muladno, C. Sumantri and S. Murtini, 2013. Association of Mx gene genotype with antiviral and production traits in Tolaki chicken. Int. J. Poult. Sci., 12: 735-739.
36. Ramasamy, K.T., M.R. Reddy, K.S.R. Ravindra and R.N. Chatterjee, 2018. Chicken Mx gene polymorphisms in Indian native chicken breeds and white leghorn by real time multiplex allele specific PCR. Indian J. Anim. Res., 56: 649-651.