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Structural Characterization and Biological Function Annotation of Cluster of Differentiation 14 Gene of Crossbred Cattle - An *In silico* Approach

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ABSTRACT

Cluster of Differentiation 14 (CD14) gene has been considered as an important candidate gene for its association with various disease resistance or susceptibility in several livestock species. For better understanding of the molecular mechanism of CD14, the present study aims to conduct a detailed *in silico* analysis of this protein in Karan Fries (KF) cattle in terms of its physicochemical properties, three dimensional (3D) structure predictions, interacting partners and phylogenetic relationship with other orthologs. Major part of this protein is alpha helix and random coil, making it suitable for interaction with other proteins. The 3D protein structures were predicted using Modeller 10.3 and Swiss Model and validated by Ramachandran plot. Protein-protein interaction suggested that the protein may act as a cell surface receptor and play a crucial role in regulating innate immune response along with 10 potential interaction partners. Sequence based phylogenetic tree analysis showed the KF CD14 gene has close evolutional relationship with that of *Bos taurus* and *Bos indicus* cattle. The structural characterization and the predicted 3-D model of this protein will not only help in providing comprehensive idea on its molecular mechanism of action but also act as a base for further understanding its other functional potential.

HIGHLIGHTS

- Karan Fries CD14 protein sequence was retrieved from the NCBI database and subjected to *in silico* analysis.
- Three dimensional structures were predicted using Modeller 10.3 and Swiss Model.
- Protein-protein interaction analysis showed potential interaction partners of CD14, which plays a vital role in innate immune response.

Keywords: Karan Fries, CD14, Phylogenetic analysis, Protein-protein interaction

India ranks first in milk production with 209.96 Million tonnes per annum, which accounts for 22.6% of the Global milk production (927.8 Million tonnes). Crossbred cows alone contribute 29.55% of the country's total milk production with the average milk yield per day of 6.62 kg as compared to indigenous/non-descript (2.72 kg) (BAHS, 2021). Karan Fries (KF) cattle was developed at Indian Council of Agricultural Research-National Dairy Research Institute (ICAR-NDRI), Karnal, Haryana by crossing Holstein Friesian bulls with Tharparkar cows under the crossbreeding project started in 1971, wherein it was finally declared as a specific crossbred strain in 1982. Present level of Holstein inheritance in Karan Fries crossbred cattle is 62.5% (Singh and Gurnani, 2004).

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Cluster of Differentiation 14 (CD14), a glycosylphosphatidylinositol-anchored surface protein is considered as the first described pattern recognition receptor (PRR) and Opsonin receptor (Wright, 1995), expressed on the surface of monocytes, macrophages and polymorphonuclear leucocytes. CD14 being the PRR recognises Pathogen-associated molecular patterns (PAMPs), a component of the bacterial cell wall (i.e., Lipopolysachharides – LPS and peptidoglycan), specialized bacterial proteins (i.e. Flagellin), as well as nucleic acid structures unique to bacteria and viruses (i.e. CpG DNA, dsRNA) and regulates innate immune response against pathogens and other exogenous factors (Liu *et al.,* 2012). Molecular characterization of CD14 gene of Karan Fries cattle revealed its genomic size as 2630 base pairs (National Centre for Biotechnology Information Accession number - MK236490.1) and comprises of 2 exons and an intron (Selvan *et al.,* 2016).

CD14 gene is associated with numerous disease resistance or susceptibility viz. Bovine tuberculosis (Xue *et al.,* 2018), Trypanosomiasis (Morenikeji *et al.,* 2020), *Haemonchus contortus* (Rawat *et al.,* 2021), Mastitis (Ibeagha *et al.,* 2008; Gupta *et al.,* 2018; Selvan *et al.,* 2014; Kumar *et al.,* 2014) and fertility traits (Ortega *et al.,* 2017) in livestock species.

Considering the importance of CD14 gene and its proven role as a candidate gene in disease resistance or susceptibility traits in various livestock species, an attempt has been made in the present study to accurately predict the primary structure, three dimensional structure and Protein-Protein interaction network through *in silico* study in Karan Fries cattle.

MATERIALS AND METHODS

Retrieval and analysis of KF CD14 protein

CD14 protein sequence (QDG00778.1) was retrieved from the available complete CD14 gene sequence of Karan Fries cattle (MK236490.1) in NCBI database. Physicochemical properties of CD14 protein sequences was analysed (Gasteiger *et al.,* 2007) by Prot-Param tool (http://web.expassy.org/prot param/). The hydrophobic and hydrophilic nature of KF CD14 amino acids was determined by Hydropathy plot (https://web.expasy.org/protscale/). The secondary structure of KF CD14 protein was predicted by using Self-optimised Prediction Method (SOPMA) (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html) (Geourjon and Deleage, 1994). Reference CD14 protein sequence of 12 species *viz. Bos indicus* (XP_019820613.1), *Bos taurus* (ABV68571.1), *Bos mutus* (XP_005900287.1), *Bison bison* (XP_010848916.1), *Bubalus bubalis* (AVZ46791.1), *Oryx dammah* (XP_040095843.1), *Ovis aries* (ABI95799.1), *Capra hircus* (NP_001348576.1), *Sus scrofa* (ABC84487.1), *Camelus bactrianus* (XP_010970054.1), *Camelus dromedaries* (XP_010989026.1), *Equus caballus* (NP_001075396.1), *Equus asinus* (XP_044636987.1) and *Gallus gallus* (NP_001132950.1) were also queried in NCBI protein database. The retrieved protein sequences were aligned by ClustalW (Thompson *et al.,* 1994) and based on the lower Bayesian Information Criterion (BIC) score, the suitable model for constructing the phylogenetic tree was identified using maximum likelihood method for 1000 bootstrap values in Molecular Evolutionary Genetics Analysis (MEGA) software Version 11 (Tamura *et al.,* 2021). Evolutionary divergence between the CD14 protein sequences of different species was also analysed.

Prediction of three dimensional structure of KF CD14 protein

The three dimensional structural models were generated using Modeller 10.3 and Swiss Model. The Modeller 10.3 predicted ten numbers of three dimensional (3D) structural forms using Homo sapiens CD14 protein (4GLP) as template, which was selected based on maximum identity and query coverage. Among the predicted 3D models, the best possible structure was selected considering the lowest Discrete Optimized Protein Energy (DOPE) score. Swiss Model provides the best 3D model based on the properties of the target-template alignment like sequence identity and similarity, agreement between predicted secondary structures (Biasini *et al.,* 2014). These predicted protein models obtained from softwares were visualized using RasWin Molecular Graphics Windows Version 2.7.5.2. The validation of the protein structures were done by Ramachandran plot depicted using Volume Area Dihedral Angle Reporter (VADAR) Version 1.8 (http://vadar.wishartlab.com/) (Willard *et al.,* 2003). Identification of different receptor regions in the KF CD14 protein structure with respect to hydrophobicity, hydrogen

bonds, interpolated charges, aromatic receptor surface and ionizability was carried out using BIOVIA Discover Studio Visualizer.

Identification of Protein-Protein interaction network

The Search Tool for the Retrieval of Interacting Proteins (STRING - https://string-db.org/) was used for identifying the Protein-Protein interaction (PPI) of CD14 protein (Franceschini *et al.,* 2013).

RESULTS AND DISCUSSION

Karan Fries CD14 protein sequence analysis

Karan Fries CD14 protein comprises of 373 amino acid residues (Table 1) with 39.63 kDa molecular weight, Isoelectric point (pI) value of 5.37, instability index of 40.52, aliphatic index of 102.06 and a Grand average of hydropathicity (GRAVY) of 0.106 indicates that the protein is polar in nature. Analysis of various physicochemical parameters of KF CD14 protein sequences indicates that, the pI value (5.37) was less than 7 indicating its acidic nature, the instability index (40.52) of above 40 predict its instability, the higher aliphatic index (102.06) due to presence of aliphatic side chains like alanine, valine, isoleucine, and leucine may be regarded as a positive factor for increasing the thermostability of protein. The positive value (0.106) of GRAVY indicates that the protein is polar and hydrophobic in nature. The polar and hydrophobic nature of CD14 protein makes it a putative binding site for LPS having long carbohydrate chain which is hydrophilic and negatively charged (Kitchens and Munford, 1995).

Table 1: Amino acid composition of the Karan Fries CD14 protein

Sl. No.	Amino acids	Quantity	Percentage
1	Ala (A)	43	11.5
2	Arg (R)	20	5.4
3	Asn (N)	12	3.2
4	Asp (D)	20	5.4
5	Cys (C)	11	2.9
6	Gln (Q)	15	4.0
7	Glu (E)	16	4.3
8	Gly (G)	26	7.0
9	His (H)	3	0.8
10	Ile (I)	4	1.1
11	Leu (L)	64	17.2
12	Lys (K)	11	2.9
13	Met (M)	6	1.6
14	Phe (F)	9	2.4
15	Pro (P)	30	8.0
16	Ser (S)	28	7.5
17	Thr (T)	21	5.6
18	Trp (W)	5	1.3
19	Tyr (Y)	4	1.1
20	Val (V)	25	6.7

The Hydropathy plot was plotted based on Kyte-Doolittle scale with the amino acid sequences in the X axis and the degree of hydrophobicity and hydrophilicity plotted on Y axis (Fig. 1). The plot reveals the highest hydrophobicity nature (2.689) of amino acid Isoleucine at the 11th and 12th position, whereas the Arginine at the 313th position has the highest hydrophilicity nature (-2.600). Protein secondary structure of KF CD14 gene suggested the presence of 163 alpha helices (43.7%), 17 beta turns (4.56%), 38 extended strands (10.19%) and 155 random coils (41.55%) (Fig. 2). Secondary structure comprises majority of alpha helix and random coils, elucidating their crucial role in folding stability and ligand binding (Kim *et al.,* 2005).

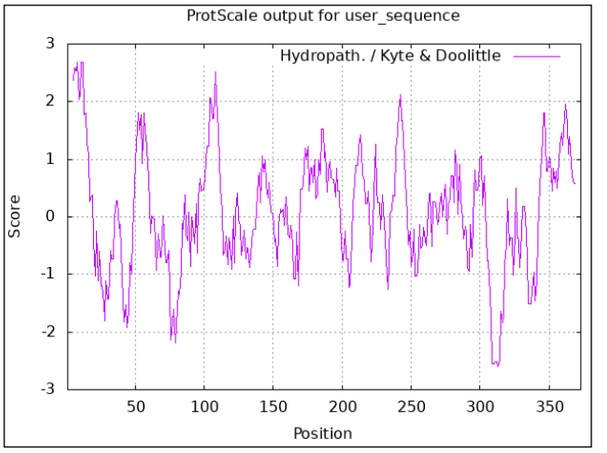

Fig. 1: Hydropathy plot for the KF CD14 protein

The protein sequence from 12 different species were aligned and Jones-Taylor-Thornton (JTT) alongwith Gamma distribution (G) model having lowest BIC score of 7135.69 was found to be best model to construct

Table 2: Amino acid sequence divergence of CD14 protein across different species

Species	Karan Fries	*Bos indicus*	*Bos taurus*	*Bos mutus*	*Bubalus bubalis*	*Oryx dammah*	*Ovis aries*	*Capra hircus*	*Bison bison bison*	*Sus scrofa*	*Equus caballus*	*Camelus bactrianus*	*Camelus dromedarius*	*Gallus gallus*	*Equus asinus*
Karan Fries		0.003	0.003	0.003	0.009	0.012	0.013	0.011	0.004	0.041	0.041	0.037	0.037	0.306	0.041
Bos indicus	0.003		0.003	0.000	0.009	0.012	0.012	0.011	0.003	0.040	0.042	0.037	0.037	0.307	0.042
Bos taurus	0.003	0.003		0.003	0.009	0.012	0.013	0.011	0.004	0.041	0.042	0.037	0.037	0.309	0.042
Bos mutus	0.003	0.000	0.003		0.009	0.012	0.013	0.011	0.003	0.040	0.042	0.037	0.037	0.307	0.042
Bubalus bubalis	0.031	0.028	0.031	0.028		0.012	0.013	0.012	0.009	0.041	0.042	0.038	0.037	0.311	0.042
Oryx dammah	0.055	0.052	0.055	0.052	0.052		0.010	0.008	0.012	0.039	0.040	0.036	0.035	0.309	0.040
Ovis aries	0.055	0.052	0.055	0.052	0.058	0.031		0.007	0.012	0.039	0.040	0.037	0.036	0.304	0.040
Capra hircus	0.044	0.041	0.044	0.041	0.048	0.020	0.014		0.012	0.038	0.040	0.036	0.036	0.298	0.041
Bison bison bison	0.006	0.003	0.006	0.003	0.028	0.050	0.047	0.042		0.043	0.043	0.040	0.040	0.330	0.043
Sus scrofa	0.318	0.316	0.319	0.316	0.324	0.320	0.322	0.312	0.313		0.041	0.038	0.037	0.306	0.042
Equus caballus	0.349	0.353	0.355	0.354	0.347	0.327	0.333	0.327	0.344	0.365		0.039	0.038	0.306	0.004
Camelus bactrianus	0.297	0.293	0.297	0.294	0.299	0.288	0.288	0.280	0.299	0.291	0.333		0.003	0.311	0.040
Camelus dromedarius	0.292	0.288	0.293	0.290	0.294	0.284	0.283	0.276	0.294	0.286	0.328	0.003		0.309	0.039
Gallus gallus	2.707	2.726	2.741	2.726	2.745	2.752	2.735	2.673	2.795	2.666	2.676	2.689	2.681		0.308
Equus asinus	0.349	0.354	0.356	0.355	0.348	0.328	0.334	0.332	0.345	0.371	0.006	0.340	0.335	2.693	

phylogenetic tree. It revealed that KF CD14 protein was more closely related to *Bos taurus, Bos indicus*, *Bubalus bubalis* than *Ovis aries* and *Capra hircus* while *Equus caballus* and *Equus asinus* were the farthest species (Fig. 3).

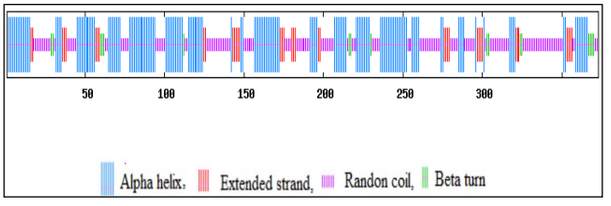

Fig. 2: Secondary structure of KF CD14 protein

Evolutionary divergence analysis of CD14 protein revealed maximum diversity between KF and Chicken, Horse and Donkey (Table 2). Phylogenetic analysis of KF CD14 protein indicates its close association with *Bos taurus* and *Bos indicus* cattle than any other species, the reason being this crossbred cattle having higher exotic inheritance of Holstein Friesian and indigenous inheritance of Tharparkar. Protein sequence divergence analysis revealed the KF CD14 sequence diverges from other species with chicken as the distant one followed by horse, donkey and pig among 13 species under consideration.

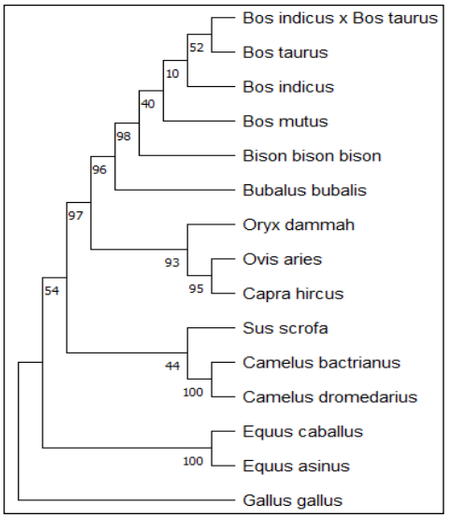

Fig. 3: Phylogenetic tree of CD14 protein across 12 animal species

Three dimensional structure of KF CD14 protein

The DOPE score of 10 different structure models predicted by Modeller 10.3 was plotted (Fig. 4) and the best prototype (Model 10) was selected based on the lowest DOPE score(-31926.82).

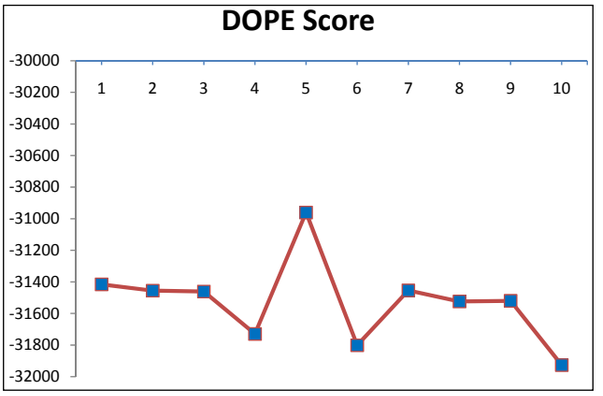

Fig. 4: Graphical presentation of DOPE scores of 10 predicted models obtained in Modeller 10.3

The three dimensional structure obtained by both Modeller 10.3 and Swiss Model were visualised using RasMol viewer (Fig. 5).

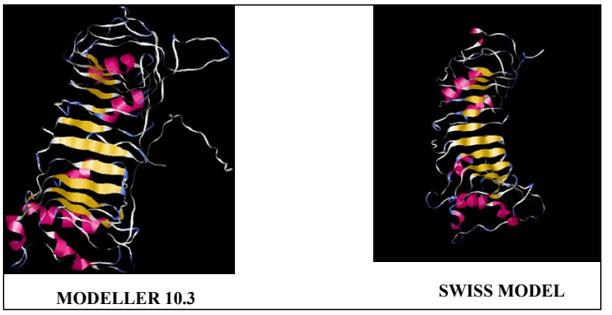

Fig. 5: Predicted three dimensional protein structures using softwares

The best prototype (Model 10) was validated using Ramachandran plot, which visualized 76.68% residues in most favoured region, 20.10% residues in allowed regions, 1.61% residues in generously allowed regions and 1.61% residues in disallowed regions (Fig. 6). Based on lowest DOPE score, the best suited prototype was selected from the Modeller 10.3 and further subjected to validation by Ramachandran Plot using the ϕ (phi) and ψ (psi) backbone torsion angles for each residue in the protein sequence (Perdih *et al.,* 2012).

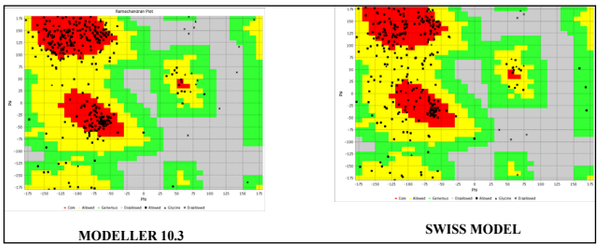

Fig. 6: Ramachandran plots of obtained three dimensional protein structures

Protein stability was validated by amount of amino acids falling under the most favoured regions of the plot. In this study, 76.68% residues fall under the most favoured regions, indicating its unstable nature.

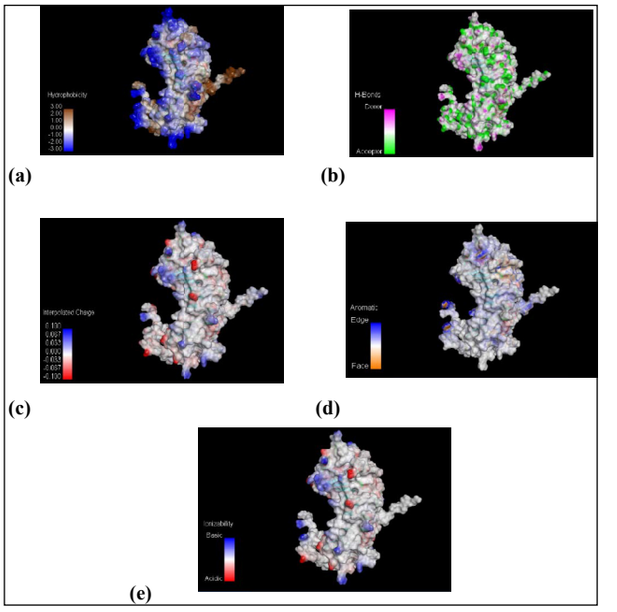

Fig. 7: Different receptor regions in the CD14 protein structure with respect to **(a)** Hydrophobicity; **(b)** Hydrogen bonds; **(c)** Interpolated charges; **(d)** Aromatic receptor surface; **(e)** Ionizability

This was in accordance with its instability (40.52) index value. CD14 protein exhibits more of polar charged receptor sites when visualized for interpolated charges receptors, which was in concordance with positive GRAVY

value (0.106). Various receptors present on the KF CD14 protein were visualised by BIOVIA Discover Studio Visualizer. The hydrophobic and hydrophilic receptors of CD14 protein were shown based on the colour ranging from brown to dark blue (Fig. 7a). Different donor and acceptor sites of hydrogen bonds (Fig.7b) and interpolated charges in the CD14 protein structure ranging from +1 to -1 (Fig. 7c) with different colour configurations were traced out. Different aromatic receptors surface present on the edges, face or intermediate regions (Fig. 7d) and evenly distributed acidic /basic ionizability regions (Fig. 7e) were represented with variable colours.

Protein-Protein interaction (PPI) network analysis

PPI analysis showed a hub of 10 potential interaction partners of CD14 (Fig. 8) viz. CD180 (Cluster of Differentiation 180), CD44 (Cluster of Differentiation 44), ITGB2 (Integrin beta 2), LY96 (Lymphocyte antigen 96), MyD88 (Myeloid Differentiation Primary-Response Protein 88), PTPN11 (Protein Tyrosine Phosphatase Non-Receptor Type 11), SPI1 (Spi-1 Proto-Oncogene), TLR2 (Toll like receptor 2), TLR4 (Toll like receptor 4) and TYROBP (TYRO protein tyrosine kinase binding protein).

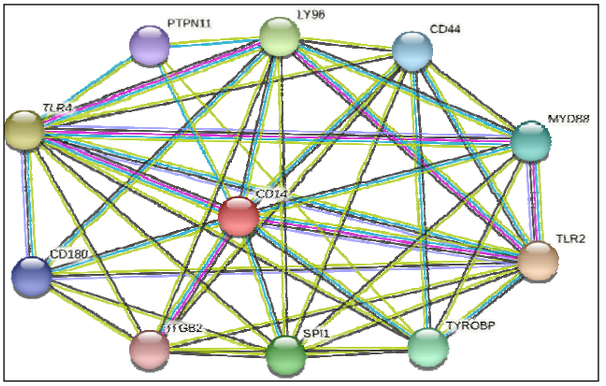

Fig. 8: Predicted potential interaction partners of CD14 protein

The complete interaction network was found to have an average node degree of 8 with 11 nodes and 44 edges. Further, Gene ontology analysis (GO) revealed that, the CD14 protein was found to be involved in 30 biological process, 5 Molecular functions and 2 Cellular components. Interaction analysis using STRING unveiled that, CD14 was connected to a network of 10 genes viz. CD180, CD44, TLR4, MyD88, TLR2, PTPN11, LY96, SPI1, TYROBP and ITGB2 with high confidence score of C0.852. Interactions with confidence score greater than 0.70 was considered as high confidence score (Franceschini *et al.,* 2013). Cluster of Differentiation family genes like CD14, CD180 and CD44 along with the lipopolysaccharide-binding protein (LBP) actively involves in cascading the reaction of innate immune response against the bacterial PAMPs (McDermott and O"Neil, 2004). TLR2 recognises bacterial peptidoglycans, while TLR4 recognises Gram negative bacterial LPS. Both TLR2 and TLR4 have an affinity to CD14 making it a potential polyspecific receptor with manifold recognition potential for broader range of pathogens (Langrova *et al.,* 2008). CD14 together with LBP plays an key role in binding LPS to Toll-like Receptor 4 and Lymphocyte antigen-96 complex (TLR4/LY96 complex) (Akashi et al., 2003). This complex in turn induces signalling cascade downstream of MyD88 activation leading to the nuclear translocation of Nuclear factor-Kappa B (NF-kB) resulting in release of proinflammatory cytokines.

CONCLUSION

The present *in silico* characterization of KF CD14 revealed that it was an acidic, polar, thermostable and hydrophobic protein having 373 amino acids localized mainly in the surface of immune cells such as monocytes, macrophages. The secondary structure analysis indicated the presence of majority of alpha helix and random coils. The protein-protein interaction analysis suggested that CD14 interacted with several other proteins involved in various biological functions like regulation of cytokine production, Toll signalling pathway, response to exogenous and endogenous stimulus suggesting the possible role in innate immune responses. These findings along with predicted 3D model will be useful in the future to encourage deeper understanding of its mechanism of action in mediating first line of defense against various infectious diseases affecting the livestock.

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