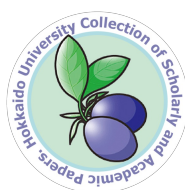




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Molecular characterization of the *lymphocyte activation gene-3 (LAG-3, CD223)* of swamp- and riverine-type water buffaloes (*Bubalus bubalis*)

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Abstract

The present study was conducted to characterize *LAG-3* of swamp- and riverine-type water buffaloes by DNA sequencing, homology and phylogenetic analysis. Bubaline *LAG-3* sequence contained an open reading frame of 1551 nucleotide, encoding a polypeptide of 516 amino acids. Nucleotide and amino acid sequence homology of *LAG-3* revealed 76–96% and 61–94% identity in water buffalo to that of other mammals, respectively. *LAG-3* protein sequence of water buffalo contained four extracellular domains, a transmembrane domain and different conserved regions. There were three N-glycosylation sites, two sequence motifs: ‘RGD’ and ‘WXC’ motif and five cysteine residues located at different positions of extracellular region. Likewise, the possible serine phosphorylation site and the ‘KTGELE’ inhibitory motif were found in the intracellular region of bubaline *LAG-3*. However, one highly conserved cysteine residue in mammalian *LAG-3* was replaced by tyrosine in both swamp- and riverine-type water buffaloes. Phylogenetic analysis generated high bootstrap value between the two types of water buffalo which further confirmed the degree of relationship between bubaline species. This was the first report that describe the genetic characteristic of *LAG-3* in swamp- and riverine-type water buffaloes.

Key Words: *LAG-3*; swamp-type water buffalo, riverine-type water buffalo, phylogenetics

Introduction

Inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), *Lymphocyte activation gene-3 (LAG-3)*, and programmed cell death 1 (PD-1, CD279)

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function at multiple levels to ensure appropriate T-cell homeostasis, activation, and differentiation^{7,13,26,27,28}. These inhibitory molecules also contribute to cell extrinsic regulation by controlling T_{reg} homeostasis and function, mediating induced T_{reg} development, and mitigating dendritic cell differentiation and function^{7,13,26,27,28}.

LAG-3 (*CD223*) is a member of the immunoglobulin superfamily and has been identified as a membrane protein and is expressed on various immune cells²⁶. *LAG-3* is involved in the down-regulation of immune responses during the progression of chronic diseases, as well as in facilitating immune evasion by several pathogens causing chronic infections and tumors^{8,14}. Human and murine *LAG-3* were mainly expressed in activated T-cells and natural killer (NK) cells⁸ while bovine *LAG-3* was mainly expressed in $CD4^+$ and $CD8^+$ T-cells²³. The complete nucleotide sequence of *LAG-3* was found to be between 500–2,000 bp in some representative animals.

Comparative in-depth study of the molecular structures of certain genes involve in the cell mediated immune responses between two closely related species is important to determine differences with regards to their reaction to pathogens. Transcriptional studies of these important proteins are also vital to elucidate their expression in the event of infection^{15,20}. Molecular analysis could provide valuable information to be used to study the role of *LAG-3* and other inhibitory receptor molecules in an organisms' immune system^{15,18,19}. A pre-investigated query gene with well-established functions or roles can be used to study the function of a similar gene from the same species or related organisms⁴. A study conducted about the murine *LAG-3* sequence wherein the removal of a highly conserved motif found on the cytoplasmic region completely abolished the function of the gene²⁶. On the other hand, the over expression of *LAG-3* that was observed in cattle infected with bovine leukemia virus and mice with lymphocytic choriomeningitis virus

implies its apparent involvement in negatively regulating T cell proliferation during chronic disease progression¹⁶.

There are still no studies conducted regarding the genetic characteristic of *LAG-3* in bubaline species. Fortunately there is already existing data in the sequence of *LAG-3* in cattle. Cloning and sequencing of bovine *LAG-3* have an open reading frame of 1,551 nucleotides, encoding a polypeptide of 515 amino acids in length²³. Since both bovine and bubaline are closely related species, bovine *LAG-3* sequence served as the reference for the determination of the genetic characteristic of bubaline *LAG-3*. The present study aimed to establish the *LAG-3* sequence in water buffalo. This study can contribute to the understanding of the structure of the immune responses and provide clues for immunotherapeutic strategies. Thus, it can help to improve the health of water buffaloes to certain diseases. Moreover, it can increase the productivity and efficiency of the agriculturally significant animal. On the other hand, the differences that may arise between the *LAG-3* of swamp- and riverine-type water buffaloes can contribute to their speciation. This present study was conducted in order to determine and describe the sequence of the *LAG-3* in swamp- and riverine-type water buffaloes.

Materials and Methods

Clinical samples: Ten representatives from each type, Murrah buffalo (riverine-type) and Carabao (swamp-type) were selected for the collection of samples. The age of the selected animals were one year and above. Murrah buffalo and Carabao were raised on communal confinement and in backyard housing, respectively. Blood was collected in sterile tubes with lithium heparin to prevent coagulation. After collection, the samples were prepared for subsequent peripheral blood mononuclear cell (PBMC) culture.

PBMC isolation and cultivation: Bubaline PBMCs were isolated from collected heparinized venous blood by density gradient centrifugation with percoll (Sigma-Aldrich, St. Louis, MO, USA). In a 50 ml tube, 10 ml of fresh blood and 40 ml of EDTA PBS pH 7 was added and was centrifuged at $1,790 \times g$ for 15 min. After centrifugation, 10 ml of buffy coat was transferred in a 15 ml tube containing 4 ml of 60% percoll solution. The tube was again centrifuged for 20 min at $1,030 \times g$. PBMC was then collected and transferred in another 15 ml tube with 15 ml EDTA PBS. The cell suspension was subjected to centrifugation at $150 \times g$ for 10 min. The solution was washed with EDTA PBS twice after centrifugation and the supernatant was discarded. Suspension of the cell was done in 1 ml of Rowell Park Memorial Institute medium 1640 (RPMI) (Gibco BRL, Grand Island, NY, USA) at room temperature. Concanavalin A was added RPMI with PBMC and cultured for 12–24 hr. The culture was transferred to a 5 ml centrifuge tube and was centrifuged at maximum speed for 10 min. Cell lysis solution was added after the supernatant was discarded. The solution was transferred in another centrifuge tube and was centrifuged twice at $19,320 \times g$ for 1 min.

RNA extraction: Total RNA was extracted from cultivated PBMC using TRIzol® reagent (Invitrogen, Carlsbad, Ca, USA) according to the manufacturer's instructions. First, the PBMCs were homogenized through the addition of 1 ml TRIzol® Reagent. After homogenization, different phases of the sample were separated. The samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. Then, 0.2 ml of chloroform was added. The tube was shaken vigorously by hand for 15 seconds and was incubated for 2–3 min at room temperature. After incubation, the sample was centrifuged at $12,000 \times g$ for 15 min at 4°C to be able to separate the different phases. The aqueous phase was transferred into a new tube for RNA isolation.

For the isolation of RNA, the aqueous phase was added with 0.5 ml of 100% isopropanol to the aqueous phase, per 1 ml of TRIzol® and was incubated at room temperature for 10 minutes to precipitate the RNA. The aqueous phase was centrifuged at $12,000 \times g$ for 10 min at 4°C. The pellet was washed with 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent. The sample was briefly subjected to vortex and was centrifuged at $7,500 \times g$ for 5 min at 4°C. After centrifugation, the wash was discarded and the RNA pellet was air dried for 5–10 min. RNA pellet was resuspended in 50 µl RNase-free water. RNA samples were refrigerated at –20°C.

Complementary DNA (cDNA) synthesis: The cDNA synthesis was done using cDNA Synthesis Kit (Takara Bio Inc.) following the manufacturer's procedure. RNA primer mixture was prepared using 1 µl of random hexamers, 1 µl of dNTP, 3 µl of extracted RNA templates and 5 µl of RNase free DW. The mixture was incubated in a PCR machine at 65°C for 5 min and was cooled immediately on ice.

Reaction mixture was prepared containing 4 µl of 5x PrimeScript Buffer, 0.5 µl of RNase Inhibitor, 1 µl of PrimeScript RTase and 10 µl of RNase free DW. The total reaction mixture was first incubated at 30°C for 10 min. The temperature was then raised to 50°C for 45 min and raised further to 95°C for another 4 min. After incubation, total reaction mixture was stored at 4°C until subsequent reaction.

β -actin gene amplification: The β -actin primer sequences: forward 5'-CGC ACC ACC GGC ATC GTG AT-3' and reverse 5'-TCC AGG GCC ACG TAG CAG AG-3'²⁴⁾. A total volume of 18.1 µl that contains 12.5 µl Top Taq, 1.6 µl MgCl₂, 0.5 µl of 10 pmol forward and reverse primers and 3.0 µl of DNA template were prepared.

The β -actin primer set (F/R) PCR profile cycle was: initial denaturation for 5 min at 94°C, 35 cycles of denaturation step for 30 sec at 94°C, annealing step for 30 sec at 55°C, and extension

step for 30 sec at 72°C followed by final elongation step for 5 min at 72°C that completed the reaction. Amplicon size was ~227 base pairs. Only samples positive for β -actin gene was further subjected to PCR.

LAG-3 gene amplification: The LAG-3 primer pair is: forward 5'-ATG CTG TGG GAG GCT TGG TT-3' and 5'-TCA GGG TTG CTC TGG CTG CA-3'²³. PCR was carried out in a total volume of 20.0 μ l containing 6.9 μ l of DDW, 4.0 μ l of 10x PCR buffer, 1.4 μ l of MgCl₂, 1.6 μ l of dNTPs, 0.5 μ l each of 10 pmol primers, 0.1 μ l of Taq polymerase, and 5.0 μ l of DNA template. A negative control containing DDW instead of DNA template was used.

The PCR cycle profile for the LAG-3 primer set was 1 cycle of initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by another extension step at 72°C for 5 min to complete the reaction. Amplicon size was 1,551 bp.

All PCR products were analyzed using 2% agarose gel and viewed under short UV illumination.

DNA sequencing: PCR products were sent to First Base, Malaysia for DNA sequencing.

Homology analysis: Homology analysis of LAG-3 nucleotides of swamp- and riverine-type water buffalo were done using Basic Local Alignment Search Tool (BLAST) program. CLUSTAL X program was used for the multiple sequence alignment. The locations of signal peptide cleavage site, transmembrane helix, and N-Glycosylation sites in the deduced amino acid sequences were determined using Signal P4.1, TMHMM v.2.0, and NetNGlyc 1.0

Phylogenetic analysis: Phylogenetic analysis was performed using the MEGA 5.0 program. The evolutionary history was inferred using the neighbor-joining method.

Results

Homology analysis

The sequence of LAG-3 of both types of water buffaloes encompassed 1,551 nucleotides encoding 516 amino acids. The LAG-3 nucleotide sequences of both types of water buffaloes were 95% identical. The sequence identity of water buffalo LAG-3 nucleotide sequence with other mammalian species is 76–96% while its deduced amino acid sequence is 61–94% (Table 1). High homology in LAG-3 sequences among different

Table 1. Nucleotide and amino acid homology percentages of LAG-3 of different mammals with reference to bubaline species

Species	Maximum Identity (%)			
	Swamp-type (LC033567)		Riverine-type (LC033568)	
	Nucleotide	Amino Acid	Nucleotide	Amino Acid
Cattle (NM001245949)	94	91	96	94
Goat (XM005681013)	90	77	94	81
Sheep (XM004007581)	91	74	95	77
Horse (XM001492398)	81	68	81	71
Pig (NM001105306)	84	76	85	78
Human (NM002286)	79	68	82	70
Mouse (NM008479)	79	61	76	63

(GenBank Accession Number)

mammalian species including both types of water buffaloes was revealed and may imply that this gene have little divergence in bubaline species.

There were nine amino acid substitutions between the *LAG-3* sequences of bubaline species and 26 amino acid substitutions between swamp-type and Artiodactyl species located at different positions (Fig. 1). The predicted signal peptide cleavage site by SignalP was located at amino acid residue 23 and 24. The amino acid residues 1–23 represent a leader peptide region. Amino acid residues 24–434, 435–473, 432–441 and 474–536 corresponds to extracellular domain, transmembrane domain, possible cleavage sites by metalloproteases and cytoplasmic region, respectively (Fig. 1).

The extra loop in Domain 1 of swamp- and riverine-type water buffalo *LAG-3*, similar to that of the cattle, pig, sheep and goat, showed 15 amino acid deletions. Five cysteine residues found in the extracellular region of bubaline *LAG-3* are well conserved among the mammalian species except for one cysteine residue in Domain 1 which was substituted in sheep (V). Interestingly, the highly conserved second cysteine residue found in Domain 4 of mammalian *LAG-3*, a part of the 'WXC' (Tryptophan-X-Cysteine, where X can be any amino acid residue) structural motif, is replaced by a tyrosine (Y) in swamp- and a histidine (H) residue in riverine-type water buffalo. In addition, *LAG-3* consists three possible N-linked glycosylation sites; these are NCS, NVS

	Domain 1									
Swamp	MLWEANFQVW	LFLQLLCAAA	VEAPEPGA	EV	PVVWAQEGAP	<u>AQLP</u> <u>C</u> SPTIP	LQDLSLP	TR		
Riverine										
Cattle		W...								
Goat	...D.R...	W...								
Sheep	Q...	W...								
Horse	-MRQ.Q.L.S	.L....WV.P		T.....				L.AG		
Pig	-MR..H.LI.	.L.....	D.R...					L..G		
Mouse	-MR.DLLGF	.L.G...WE.P	.VSSG..K.L		VH....LKS.	NL.PNFL.RG				
Human	-M...Q.LGL	P.WV.P	.KPLQ.....					L.RA		
	Domain 1 (extra loop)									
Swamp	QVTWQHVPES	GS-----	-----AAP	TPRGPGRRY	TVLRLAPGGL	RIGKLPLQPR				
Riverine		-----	-----							
Cattle		-----	-----							
Goat	R	YA-----	-----PQT	AMT.TPRFS-	-----	-----				
Sheep	R	YA-----	-----PQT	AMT.TPRFSD	CYSHTCIPRH	GCLSGC.EGQ				
Horse	G....L.D.	DPPGPGPSRP	SALGLRPP..	S.....V.	T...	LSRR...A.H				
Pig	L.DR	.P-----	-----	S.QS.....	V.....	...RP.....				
Mouse	G.I...Q.D.	.QPTPIPALD	LHQG---M.	S..Q.A.G..	...SV.....	.S.RQ..H.H				
Human	G.....Q.D.	.PPAAAPGHP	LAPGPHP...	SSW..R....	...SVG....	.S.R.....				
Swamp	VQLEEMGLQR	GDFSLWLRPA	RRADAGEYHA	AV--RFGNRA	LACRLRLRVG	QAAVTASPPG				
Riverine				..--.....						
Cattle				..--.....						
Goat	-----	-----	-----	-----	-----	-GN.....				
Sheep	K.RM.GHH.G	-----VAN	A.DRTLQSRG	.R--LG.G.P	IPVTP.MGL	RSSM.....				
Horse	...A.R...	G	C.	.R--LRD.V	.R.....A	RPSM.....				
Pig	R...		.D....R.	..SL.DRD.	.V.L.....					
Mouse	R...		L.T.....	T.R--LP...	.S.S.....	.SMI...S.				
Human	...D.R.R.		R.	..H--LRD..	.S.....L.	..SM.....				

Fig. 1. Deduced amino acid sequences alignment of *LAG-3* gene from swamp- and riverine-type water buffaloes compared with other species. Dots in the sequence signify the similarity of the amino acids. Dashes indicate gaps in the alignment. Cysteine residue (C), N-linked glycosylation sites, and serine residue (S) are underlined and bold. Solid circle and star indicates amino acid substitution between bubaline to other mammals & between swamp-type buffalo to other artiodactyl species. Highlighted in box are RGD and WXC sequence motif. Amino acid residues 432–441, indicated by plus (+), are possible cleavage sites by metalloproteases. Enclosed in the box is the KTGELE inhibitory motif.

	Domain 2							
Swamp	PLWTSWVVL	NCS FSRPDL	ASVHWFR--G	PGRVPVQESP	HHHLVGNFLF	LPQVSSLD	SG	
Riverine								
Cattle								
Goat				.D.....				
Sheep						S...		
Horse	.R.LD..I.			...I.....	.YFT.SL..	P...		
Pig	S....H..I.	F.			.Q.....	.S.I.P....		
Mouse	V.KL.D..L.	R.	V.....Q--	QN...YN..	R.F.AET..L	P....		
Human	S.RA.D..I.	R.	NR.	Q.....R...	...AES...	PM...		
●								
Swamp	TWGC CS LT	YRD	GF NVS I	TYNL	AVLGLEPRAP	LTVYAGAGSK	VELP C RLPPG	VGIQSSLTAL
Riverine								
Cattle								M
Goat			N.....	Q..		C.....		S.M
Sheep			N.....	Q..				M
Horse	P...I.....	..K...M...	T.....	SV.	A...R	H....	..T.....	R
Pig	...I.....		T....Q.	PV.	AP.AT	H..R.	..T.....	T
Mouse	...V.....		K.....	V..	AE..R	H....	..TP.L.I.	K
Human	P...I.....	M...	T.....	PT.	R	.G.....A.	..TR.F...	K
★ ● ★ ★★ ★ ★★ ★★								
	Domain 3							
Swamp	WTPPGGGPDL	LVVGD RDNST	LRLEAVGQGW	PGTYTSRIHL	QRRRLSSMAT	LAVITVTFGK		
Riverine		..A....F.	DQ	CC..	.G.Q..TTV.	PKP		
Cattle	E....	..A...N.F.	AQ	A....C.V..	.G.Q..ATV.	PKP		
Goat		..A...G.F.	AQ	A....C.V..	.G.Q..ATV.	PKP		
Sheep	..L.....	..A...G.F.	AQ	A....C....	.G.Q..ATV.	PKP		
Horse	.AL.....	..A..NG.F.	SLAQ	A....CC..	.GQQ..ATI.	PKS		
Pig		..A..HG.F.	SAQ	A....C.V..	.GQQ..ATV.	PKS		
Mouse	E.	P.A.KSG.F.	..H....LAQ	A....CS..	.GQQ.NATV.	PKS		
Human		..T..NGDF.	D.S.AQ	A....CH...	.EQQ.NATV.	..I....PKS		
★★★★ ★ ★★ ★ ★★ ★ ● ★ ● ●								
	Domain 4							
Swamp	SGSQVHLPKP	F CA VDPASGQ	EGCVWSPLVK	QHRRSSPGPS	LLTPDARPLF	YFPQYRLYQR		
Riverine	Y..SCSMR..	T....	.HF....D.	.YQ....A.W	S...	H.W.H....		
Cattle	Y..SGS.R..	..E.T....	.RF....D.	RSQ.R....W	S	Q.W.CH...G		
Goat	Y..SGS.R..	T....	.RF....D.	.SQ.....W	PS	R.W.C...G		
Sheep	Y..SGS.R..	..E.T....	.RF....D.	.SQ.....W	PS	Q.W.C...G		
Horse	L.FPGN.R.L	L.E.T....R	.RFM...VNE	PSL.G....W	.EVQE..L.S	Q.W.CH...G		
Pig	..LPGNPK.L	L.E.T....	.RF...S.D.	RSW.....W	.VLQE..L.S	Q.W.CQ..EG		
Mouse	F.LPGSRG.L	L.E.T....K	.RF..R..NN	-LS..C...V	.EIQE..L.A	ERW.CQ..EG		
Human	F..PGS.G.L	L.E.T.V...	.RF...S.DT	PSQ..FS..W	.EAQE.QL.S	Q.W.CQ...G		
★ +++++								
	Transmembrane							
Swamp	QRLLGTA	YVL	TELFHPGAQR	SGRALGAGRT	AHLPLLILG-	LLFLLLLVTG	AFSFLWRRQ	
Riverine		I..S.....						
Cattle	E.....	..S.....	F.....				S.....	
Goat		..S.....				F.....	R	
Sheep		..S.....				F.....	R	
Horse	E.....	..F.....	..SG.....	..G.P..LK.	GL..FLILG	I...S....	..LG...R...	
Pig	E.....	..G.F.....	..SR.....	..G....QK.	G...F....	T.L....S.	..I...V...R	
Mouse	AT..A	A.SSS-..HS	AR.IS.DLKG	G..V.VLILG	A.S.F...A.	..G..W..K.		
Human	E....A...F	..SS.....	P..LPA	G..L.FLILG	V.S.....	..G.....		
● ● ●								
	Intracellular region							
Swamp	WRPRR S ALE	HGTHPSQASS	KTGELEPELE	PEPDPEVEPE	PKPELELRPO	LQPEHP		
Riverine					ER.			
Cattle					..E..P.SQ.	Q.		
Goat		N.....			..E...SQ..	..L.Q.		
Sheep		N.....			..E..P.SQ.	Q.		
Horse	..A.....	..I..P..Q.	..IE....AQ	..TELAL..D	..EL...QP--	-----		
Pig		..P..Q.	..IE....P.	L..E..L.V.	..Q..QL---	-----		
Mouse	LLL.....	..IQ.FP.QR	..IE....R...	T.MGQ.P...	..E.Q..PE.R	QL----		
Human		Q.I..P..Q.	..IE....Q.P.	..E..P...	..E..P..QL--	-----		

and NST (Fig. 1). N-glycosylation sites are comprised of three adjacent amino acid residues, Asn-X-Thr/Ser, where X can be any amino acid residue except for Pro²⁹⁾.

The 'RGD' motif found in Domain 1 is conserved in the *LAG-3* sequences of the bubaline species including cattle.

The intracellular region of *LAG-3* contains three characteristic regions: Serine residue, KTGELE motif, EP (Glutamic Acid-Proline) repeated motif. The serine residue located at amino acid residue 487 is conserved in all mammalian species (Fig. 1). The KTGELE motif is found in amino acid residues 500–506 is conserved in *LAG-3* of the animals belonging to order *Artiodactyla*. Residues 501 and 502 corresponding to amino acids TG replaced to the IE within human, murine and horse *LAG-3* 'KIEELE' motif.

Phylogenetic analysis

The evolutionary relationship was inferred using neighbor-joining method by the MEGA5 software. Phylogenetic analysis of mammalian *LAG-3* revealed two clusters: Family *Bovidae* and Family *Muridae* (Fig. 2). The first group is consists of bubaline, bovine, ovine and caprine species while mouse is part of the second group.

The results further confirmed the degree of relationship between bubaline species. Additionally, the close relationship between water buffalo and other species of order *Artiodactyla* were emphasized. However, the *LAG-3* sequence of both types of water buffalo are most distant to that of the human and murine species. Consequently, a mammalian phylogeny constructed using 26 gene fragments of 164 mammals shows similar results.

Discussion

LAG-3 has emerged as an important immune-molecule in T cell biology. Essentially, it maintains lymphocyte homeostatic balance through various inhibitory mechanisms⁹⁾. The understanding of the structure of the immune response relies on the comparative molecular analysis of the immune receptors²²⁾. Additionally, transcriptional studies of immune receptor molecules are imperative to fully understand their expression during the events of disease and infection^{15,20)}.

High homology found in bubaline *LAG-3* sequences possibly connotes conserved biological activity of the gene in the mammalian species. Thus, it is highly probable that *LAG-3* sequences

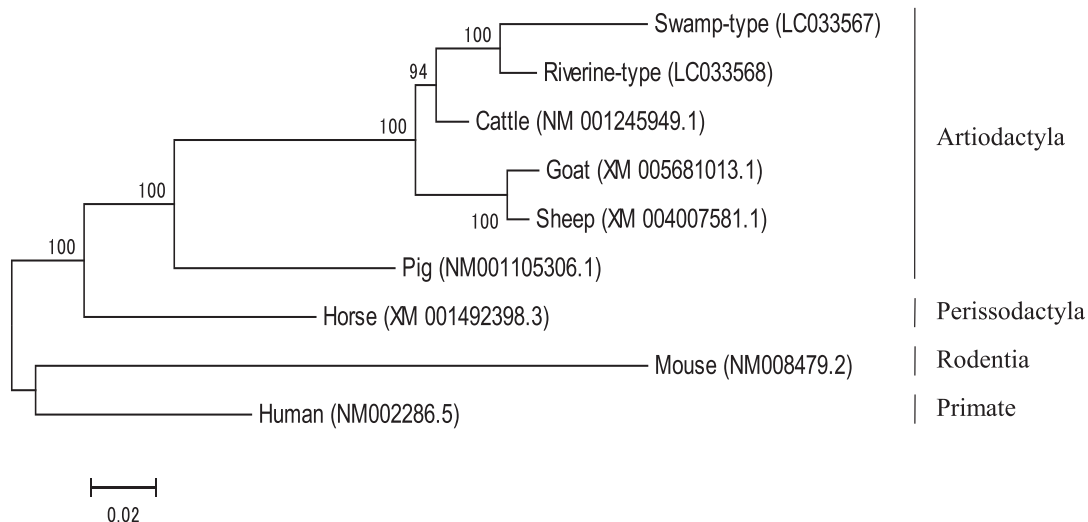


Fig. 2. Evolutionary relationship of *LAG-3* sequences of different mammalian species. Bootstrap values are based on 1000 repetitions and are shown at the nodes supporting a particular cluster.

of bubaline species have almost if not exactly the same function as the murine and bovine *LAG-3*. Bubaline *LAG-3*, like CD4, has four extracellular immunoglobulin-like domains containing conserved structural motifs throughout¹⁾. Clustered at the base of the extra loop in the first domain of the extracellular region of *LAG-3* are most of the residues essential for adhesion to MHC II proteins. The second domain is either involved in the binding or in positioning the Domain 1 for interaction with MHC II. Thus, this domain is important for successful binding between *LAG-3* and MHC II. However, both the third and fourth domains were irrelevant in *LAG-3* – MHC II interactions¹⁰⁾. All of the inhibitory receptors contain single transmembrane spanning regions and cytoplasmic tails ranging from 35 to 178 amino acids in length⁵⁾.

The replacement of cysteine by tyrosine and histidine in swamp-type and riverine-type may be a unique region in the *LAG-3* of water buffaloes. The 'WXC' signature motif is found in an equivalent position only in CD4 protein but not in any other immunoglobulin superfamily domain. Although, there are no strong validations about the regulatory functions of this motif, it may suggest how the gene may have originated. The presence of the 'WXC' motif in an unusual position suggests that *LAG-3* has evolved by gene duplication from a pre-existing two-domain immunoglobulin light chain-like structure²⁵⁾. Single cysteine to tyrosine transition appears to interfere with the receptor binding activity of the growth inhibitory function of myostatin, in cattle. This, however, is possibly a result of the disruption of a significant cysteine knot structure that is present only in myostatin²⁾. This distortion of cysteine residue in bubaline could also interfere with immuno-inhibitory function of *LAG-3*. Cysteine residues are subjected to oxidative posttranslational modification and play an important role in protein structure and cell response³⁾. This particular substitution in bubaline *LAG-3* could be the immunological basis of disease resistance/tolerance and susceptibility

in both types of water buffalo.

Meanwhile, N-linked glycosylation sites are the regions where sugar molecule known as glycan attaches to the nitrogen atom of the asparagine residue. This linkage is important for both the structure and function of the proteins^{11,21)}. Interestingly, the glycosylation site in domain 3 of swamp-type has replaced phenylalanine (F) by serine (S) and this could have a significant effect on biological function of *LAG-3*. On the other hand, metalloprotease cleavage in *LAG-3* controls the efficiency of T cell proliferation and cytokine production. Thus, its presence is significant to the effect of *LAG-3* during down regulation¹⁷⁾.

Similar with cattle, bubaline *LAG-3* has serine. Previous study suggests that this site is a possible phosphorylation site²⁶⁾. Phosphorylation occurs when a phosphate group attaches to an organic molecule, most commonly protein. This modification is an important mechanism for regulating protein activity. Hence, the presence of a possible serine phosphorylation site in *LAG-3* may play an important role in *LAG-3* function⁶⁾.

KTGELE/KIEELE conserved motif is significant to *LAG-3* signaling during down regulation of T cell expansion. However, study conducted regarding the functions of the motifs in the cytoplasmic tail of *LAG-3* showed that the absence of the EP repeated motif had no effect on *LAG-3* function, although it may help the KTGELE/KIEELE motif in preventing co-receptor activity. The EP amino acid repeated motif, that is quite unique in human *LAG-3*, is the binding motif between *LAG-3* and *LAG-3*-associated proteins (LAP)¹²⁾. Although EP motif is found within the bubaline *LAG-3*, it is short and interrupted by other amino acid residues. LAP's connection to *LAG-3* function has not yet been confirmed and its importance has yet to be determined²⁶⁾.

Generally, genetic characteristic of *LAG-3* in water buffaloes may have similar function to that of other closely related species. Nevertheless, the substitutions in conserved motif and other sites of *LAG-3* in water buffaloes may have an effect

in T cell homeostasis. If so, this could be one of the initial steps to the understanding of immune response and developing immuno-therapeutic strategies in water buffaloes diseases. The present study was first report that describes the genetic sequence of *LAG-3* in swamp- and riverine-type water buffalo. However, further studies are required to determine the effect of these variations since these may have impacts to the inhibitory function of *LAG-3*.

Conflict of interest

The authors declare no conflict of interest.

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