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Molecular cloning, sequencing and phylogenetic analysis of inflammatory cytokines of swamp type buffalo contrasting with other bubaline breeds

Claro N. Mingala[a,b,*], Raadan Odbileg[a], Satoru Konnai[a], Kazuhiko Ohashi[a], Misao Onuma[a]

[a] *Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan*
[b] *Philippine Carabao Center National Headquarters and Genepool, Science City of Munoz, 3120 Nueva Ecija, Philippines*

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Abstract

The current research concerned in the cloning, sequencing and phylogenetic analysis of inflammatory cytokine (IL-1α, IL-1β, IL-6 and TNF-α) genes from swamp buffalo and two bubaline breeds, CB (cross between swamp and riverine type buffalo) and the Bulgarian Murrah buffalo. Multiple sequence comparison showed a high homology between the bubaline breeds, which ranged from 99.3% to 100.0% similarity, whereas from 98.6% to 99.0% compared to cattle. The phylogenetic analysis had confirmed and justified the degree of relationship between these bubaline species and their distinctness to each other by the bootstrap value (%) generated. These findings were discussed with particular attention to the diversity of the inflammatory cytokine proteins within closely related species. The result of this study concluded that a small difference in the cytokine structures might be the reason behind

*Corresponding author. Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku Kita 18 Nishi 9 chome Sapporo, Hokkaido 060-0818, Japan. Tel.: +81 80 5588 4267; fax: +81 11 706 5217.
E-mail address: cnmingala@hotmail.com (C.N. Mingala).

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or has a contributory factor on the previous reports about the existence of disease resistance. However, in-depth study is necessary to further qualify these findings.
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Keywords: Swamp buffalo; Bulgarian murrah buffalo; Crossbreed; Inflammatory cytokines; Carabao

Résumé

La recherche en cours concerne le clonage, l'enchaînement séquentiel et l'analyse phylogénétique des gènes inflammatoires de cytokines (IL-1α, IL-1β, IL-6 et TNF-α) du buffle de marais et de deux races différentes de bubalines, les hybrides (hybridation entre le buffle de type marais et celui de type riverain) et le buffle bulgare de Murrah.. La comparaison des séquences multiples a révélé une homologie élevée entre les espèces de bubalines, avec un pourcentage de similitude se rangeant entre 99.3 et 100.0%, alors que les chiffres concernant le bétail font état d'un écart de 98.6 à 99.0%. L'analyse phylogénétique avait confirmé et justifié le degré de relations entre ces espèces de bubalines ainsi que leurs différenciations par la valeur de bootstrap (%) produite. Ces découvertes ont été discutées en tenant particulièrement compte de la diversité des protéines inflammatoires des cytokines à l'intérieur de groupes d' espèces étroitement liées. Le résultat de cette étude a démontré que de petites différences dans les structures des cytokines pourraient être soit la raison de base soit un des facteurs qui contribuerait à expliquer pourquoi, dans les rapports précédents, l' existence d'une résistance à la maladie avait été constatée. Toutefois, des recherches plus approfondies sont nécessaires pour justifier davantage de telles découvertes.
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Mots clés: Buffle de marais; Buffle bulgare de Murrah; Hybridation; Cytokines inflammatoires; Carabao

1. Introduction

Inflammatory cytokines such as interleukin (IL) 1α, IL-1β, IL-6 and tumor necrosis factor (TNF)-α are produced predominantly by activated immune cells such as microglia and are involved in the amplification of inflammatory reactions [1]. They have a wide spectrum of biological activities that help to coordinate the body's response to infection. The regulation of inflammatory response at the site of infection is thought to depend in part on the release of soluble mediators that attract, focus and activate other inflammatory cells. These cytokines are also endogenous pyrogens, raising body temperature, which is believed to help eliminate infections. A major effect of these cytokines is its action on the hypothalamus, altering energy mobilization to increase the body temperature. At elevated temperature, bacterial and viral replications are decreased, while the adaptive immune response operates more efficiently [2,3].

Both of the two forms of human and murine IL-1 (alpha and beta) are non-glycosylated proteins of 17 kDa. Although the two forms are only 23% homologous [4–6], the conserved portion is assumed to represent the active site because they have identical biological activities. Meanwhile, IL-6 is a multifunctional cytokine that

plays a central role in inflammatory responses and in the regulation of cells of the hematopoietic system [7]. This cytokine belongs to the neuropoietic cytokine family, which also includes the ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, and cardiotrophin 1 (CT-1) [8,9]. Macrophages, monocytes and lymphocytes do not produce IL-6 unless appropriately stimulated by IL-1, TNF-α or viral infections [10–12]. The IL-6 acts with IL-1 to stimulate the release of acute-phase proteins from hepatocytes, although it is much less potent than IL-1 or TNF-α. On the other hand, TNF-α, aside from stimulating IL-1 and IL-6 genes, also enhances the expression of CD11b/CD18. It acts as a growth stimulator for fibroblast, has direct antiviral activity, and synergizes with interferons (IFNs). These effects are not due to the induction of IFN synthesis but by the IFN's accelerating effect on the TNF's selective killing of virus-infected cells [13].

There are two types of water buffalo, the swamp and the riverine type buffalo. Native water buffalo or carabao that is found in the Philippines and in the South and Southeast Asians region belongs to swamp type water buffaloes, whereas the rest which is mainly found in India, Europe and the Americas are of riverine type to include the Bulgarian Murrah buffalo. Swamp and riverine type water buffaloes have different chromosome number which is 48 and 50, respectively. Interestingly, their crossbreed has 49 chromosome complement [14–16].

Recently, inflammatory cytokines have already been cloned and sequenced from several mammalian species including the TNF-α and IL-6 of Indian water buffalo (riverine type) [17,18]. However, no sequence and analysis have been made yet so far in swamp type buffalo. Only recently that cloning and sequencing of Th1 and Th2 cytokines had been reported in this type of water buffalo [19].

This study was conducted to understand more of the differences in the immunological activities of these cytokines between the riverine and the swamp buffaloes. A comparison was made on the result of molecular cloning and sequencing of inflammatory cytokines in swamp buffalo, riverine buffalo and crossbred (swamp × river-type) buffalo with that of the existing sequences of other mammalian species in the GenBank. Sequence homology assessment and phylogenetic analyses were done to elaborate the distinctness of each species and to initiate research on the immunological basis behind the claim that swamp-type buffalo is more disease-resistant than its riverine counterpart [20,21].

2. Materials and methods

2.1. Preparation of water buffalo PBMCs, RNA isolation and RT-PCR

Blood samples were collected from the Philippine Carabao Center Genepool in the Philippines. Three animals from each breed, i.e. Philippine carabao (*Bubalus carabanensis*), a cross between Philippine carabao and Indian Murrah (*Bubalus bubalis*) (CB) and Bulgarian Murrah Buffalo (BMB), were chosen as blood sources. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation using Percoll method (Amersham-Pharmacia, UK). The PBMC were

incubated in RPMI 1640 medium (Gibco BRL, USA) containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 ng/ml streptomycin. Cells (1×10^7/ml) were grown for 24 h in 24-well plates and stimulated with Concanavalin A (Con A, 5 μg/ml), representing an optimal time for the stimulation determined by a previous experiment (data not shown). Total RNA was isolated from Con A-stimulated PBMCs using the TRIzol reagent (Invitrogen, USA).

An aliquot of the total RNA (5 μg) was reverse-transcribed by RAV2 reverse transcriptase (20 U/μl, TAKARA, Japan) and the oligo-dT primer (0.5 mg/ml) in total volume of 40 μl reaction according to the manufacturer's instructions. IL-1α, IL-1β, TNF-α and IL-6 cDNAs were amplified by PCR using primers designed previously for Llama cytokine cloning and some were newly designed based on bovine cytokine sequences reported in GenBank (Table 1).

PCR was carried out in total volume of 20 μl reaction buffer containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 5 U of *Taq* polymerase (Takara), 10 pmol each of the primers, and 2 μl of cDNA. Cycling conditions for PCR were 35 cycles of 45 s at 94 °C, 45 s at annealing temperatures depending on cytokines to be amplified and 1 min at 72 °C, followed by the final extension for 5 min at 72 °C. Resultant PCR products were separated on 2% agarose gel containing 0.5 μg/ml of ethidium bromide, and visualized under a UV light.

2.2. Cloning and sequencing of water buffalo inflammatory cytokine genes

The amplified bands corresponding to cytokine cDNAs were excised from the gel and purified using the Geneclean kit (Bio 101, USA). The purified cDNA fragments were ligated into the pGEM-T easy vector (Promega, USA), and transformed into a competent *E. coli* strain DH-5α. In each experiment, eight to 10 plasmid clones containing cytokine cDNAs were sequenced using the BigDye terminator cycle sequence kit and an automated DNA sequencer (PRISM 310 Genetic Analyzer, Applied Biosystems).

Table 1
Cytokine primers used in this study

Cytokines	Predicted size (bp)	Primers	Annealing temperature (°C)
IL-1α (F_1)	556	5′ ATG GCC AAA GTC CCT GAC C ′3	48
IL-1α (R_1)		5′ TTC TTA GAG TCA CAG GAA G ′3	
IL-1α (F_2)	356	5′ GCA GTG AAA TTT GAC ATG G ′3	45
IL-1α (R_2)		5′ GTC ACC ACT TTA CAA GTA AG ′3	
IL-1β (F)	805	5′ CCA TGG CAA CCG TAC CTG AAC CCA ′3	55
IL-1β (R)		5′ CTT TAG GGA GAG AGG GTT TCC ATT ′3	
IL-6 (F)	719	5′ GGA ACG AAA GAG AGC TCC ATC ′3	55
IL-6 (R)		5′ TTC TGA CCA GAG GAG GGA ATG ′3	
TNF-α (F)	748	5′ GAA CCC TTC TGA AAA AGA CAC C ′3	50
TNF-α (R)		5′ GAA GAT GCA TGT CCT GCA CCC ′3	

Table 2
Accession number in the GenBank of different mammalian species used in this study

Cytokine	Scientific name	IL-1α	IL-1β	IL-6	TNF-α
Swamp	*Bubalus carabanensis*	AB246782	AB246783	AB246784	AB246785
CB[a]		AB246790	AB246791	AB246792	AB246793
BMB[b]		AB246786	AB246787	AB246788	AB246789
Riverine	*Bubalus bubalis*	AY514120	AY514903	AY347710	AY221123
Cattle	*Bos taurus*	M37210	M37211	X57317	AF011926
Pig	*Sus scrofa*	M86730	M86725	M80258	X57321
Horse	*Equus caballus*	U92480	U92481	AF005227	AB035735
Dog	*Canis familiaris*	AF047011	Z70047	U12234	Z70046
Mouse	*Mus musculus*	NM010554	M15131	X54542	MN013693
Human	*Homo sapiens*	M15329	M15330	M54894	NM000594
Cat	*Felis catus*	AF047012	M92060	L16914	M92061

[a]Crossbreed (*B. bubalus* × *B. carabanensis*).
[b]Bulgarian Murrah buffalo.

2.3. Phylogenetic analysis

Sequence data analyses were performed using the BLAST search of the National Center for Biotechnology Information. Inflammatory cytokines of water buffalo that includes IL-1α, IL-1β, TNF-α and IL-6 nucleotides and amino acid sequences were deduced using genetic information processing software (GENETYX MAC, Version 10.1.2, Software Development Co. Ltd., Japan). The sequence data herein have been submitted to GenBank and assigned each with accession number (Table 2).

Phylogenetic analysis was performed using the CLUSTALX program. The transition/transversion rates were calculated using the PUZZLE 4.0.2 program. Bootstrapping values were calculated using the modules SEQBOOT (random number seed: 123; 100 replicates), DNADIST (distance estimation: maximum likelihood; analysis of 100 data sets), NEIGHBOR (Neighbor joining and UPGMA method; random number seed: 99; analysis of 100 data sets) and CONSENSE from the PHYLIP package, Version 3.573. The phylogenetic trees were computed with DNADIST and NEIGHBOR modules with the same parameters as above, and TREEVIEW Version 1.6.0 was used for visualization of the phylogenetic trees.

3. Result

Complementary DNA of IL-1 (α and β), IL-6 and TNF-α of swamp buffalo, CB and BMB were amplified from the mRNA isolated from ConA stimulated PBMC. These cDNAs were further amplified by PCR, purified and cloned. *E. coli* plasmid DNAs were later prepared for sequencing.

Comparison of the nucleotide and amino acid sequences of these inflammatory cytokines with that of the other published sequences revealed a high homology to riverine buffalo (Murrah) and cattle, which ranges from 99.3 to 100.0% and from

Table 3
Percent identity of nucleotide and (amino acid) sequences of various inflammatory cytokines using riverine (Indian Murrah) buffalo as reference animal

%	Swamp	CB[a]	BMB[b]	Cattle	Pig	Horse	Dog	Mouse	Human	Cat
IL-1α	99.4 (99.6)	99.6 (100.0)	99.8 (99.6)	98.8 (98.1)	88.2 (84.0)	87.7 (81.7)	81.7 (75.3)	72.6 (60.0)	81.5 (72.2)	83.3 (76.4)
IL-1β	99.8 (100.0)	99.8 (100.0)	99.8 (100.0)	98.6 (98.5)	82.6 (70.8)	77.7 (69.6)	71.2 (50.4)	71.2 (57.6)	75.2 (60.7)	76.0 (62.3)
IL-6	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	98.7 (99.0)	79.2 (64.8)	75.1 (56.7)	73.3 (54.6)	60.9 (41.7)	70.6 (52.9)	72.9 (51.7)
TNF-α	99.3 (99.1)	99.3 (99.1)	99.4 (99.1)	99.0 (99.1)	86.3 (83.3)	83.8 (77.8)	83.8 (76.3)	76.2 (73.3)	84.6 (80.3)	84.3 (81.1)

[a]Crossbreed (*B. bubalis* × *B. carabanensis*).
[b]Bulgarian Murrah buffalo.

98.6 to 99.0%, respectively (Table 3). Moreover, IL-1β and IL-6 appeared to be more conserved compared to the other two cytokines. IL-6 had 100.0% homology between the bubaline species in both of its nucleotide and amino acid sequences. TNF-α appeared to be slightly divergent compared with other cytokines.

The deduced amino acid sequences of these inflammatory cytokines were aligned with other published mammalian sequences specifically of the riverine buffalo, cattle, pig and horse. The IL-1α, IL-1β, IL-6 and TNF-α had 268, 266, 208 and 234 amino acids sequence, which deduced from 807, 801, 627 and 705 bp ORF, respectively (Fig. 1a–d). Most of the putative N-linked glycosylation sites, cysteine (Cys) residues and amino acid terminal of the mature proteins were conserved among species (Fig. 1a). However, the first potential N-linked glycosylation site in IL-1α at position 102–104 was only present in the order Artiodactyla and absent in horse. Moreover, IL-1α of swamp buffalo compared to other bubaline breeds had one different amino acid at position 46 but the same with cattle, pig, and horse. BMB, on the other hand, had only one amino acid substitution at position 160. Meanwhile, the carboxyl terminal endpoints for active proteins (tripeptide of TDF) or Thr-Asp-Phe were conserved within the two forms of IL-1 genes and between all the compared species (Fig. 1a–b, marked with solid circle).

Furthermore, the IL-6 amino acid sequence contains four conserved Cys residues and lone potential N-linked glycosylation site, which did not appear on the same position with pig and horse but only within the tribe Bovini (Fig. 1c). Interestingly, similar to the previously cloned IL-10 gene [21], TNF-α had additional amino acid found at position 63, which was Q (CAG) or Glutamine (Fig. 1d). This made the peptide longer from 233 to 234 bp compared with existing TNF-α peptide of Indian buffalo.

The phylogenetic analysis (Fig. 2) constructed a cladogram tree from the generated nucleotide sequences together with the other available sequences in GenBank, which confirmed the result shown in Table 3. Sequences of IL-1α, IL-1β and IL-6 from bubaline species produced a high bootstrap value (100%). Meanwhile, TNF-α produced only a slight difference. Moreover, a cluster was formed within the order Artiodactyla that includes the bubaline species, cattle, sheep and pig.

4. Discussion

Molecular cloning, sequencing and phylogenetic analysis of inflammatory cytokines of three related bubaline species were described in this study. IL-1α, IL-1β, TNF-α and IL-6 were compared by their sequence homology with the existing sequences in the GenBank that include Indian buffalo (riverine type), cattle and pig (where all belong to order Artiodactyla) and also with horse, which belongs to order Perissodactyla. The PBMC from the subjected animals were stimulated with ConA. Thereafter, mRNAs were extracted from it and reverse transcribed to produce cDNAs, which were later cloned. Nucleotide sequence analyses of positive clones and the subsequent production of biologically active cytokines in an *E. coli* expression system confirmed the identity of the target cytokines.

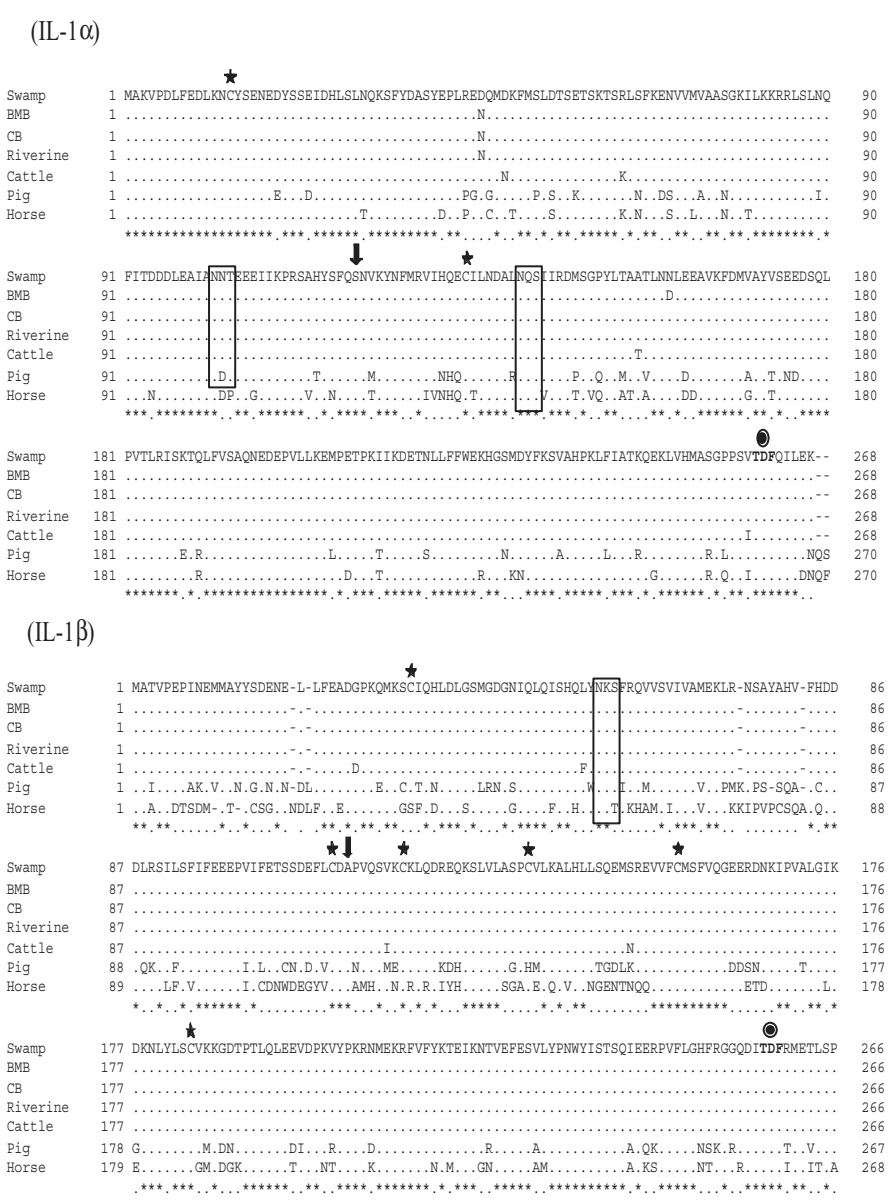

Fig. 1. (a–d). Alignment of amino acid sequences of inflammatory cytokines from swamp buffalo (*Bubalus carabanensis*), riverine buffalo (*Bubalus bubalis*), BMB, CB, cattle, pig and horse. Dashes indicate gaps in the alignment. Star indicates conserved Cysteine residues while arrow indicates amino terminal of the mature protein. Solid boxes indicate a potential N-linked glycosylation sites and the solid circle denotes carboxyl terminal end point while the solid triangle on the **Q** in (d) denotes the extra amino acid, Glutamine.

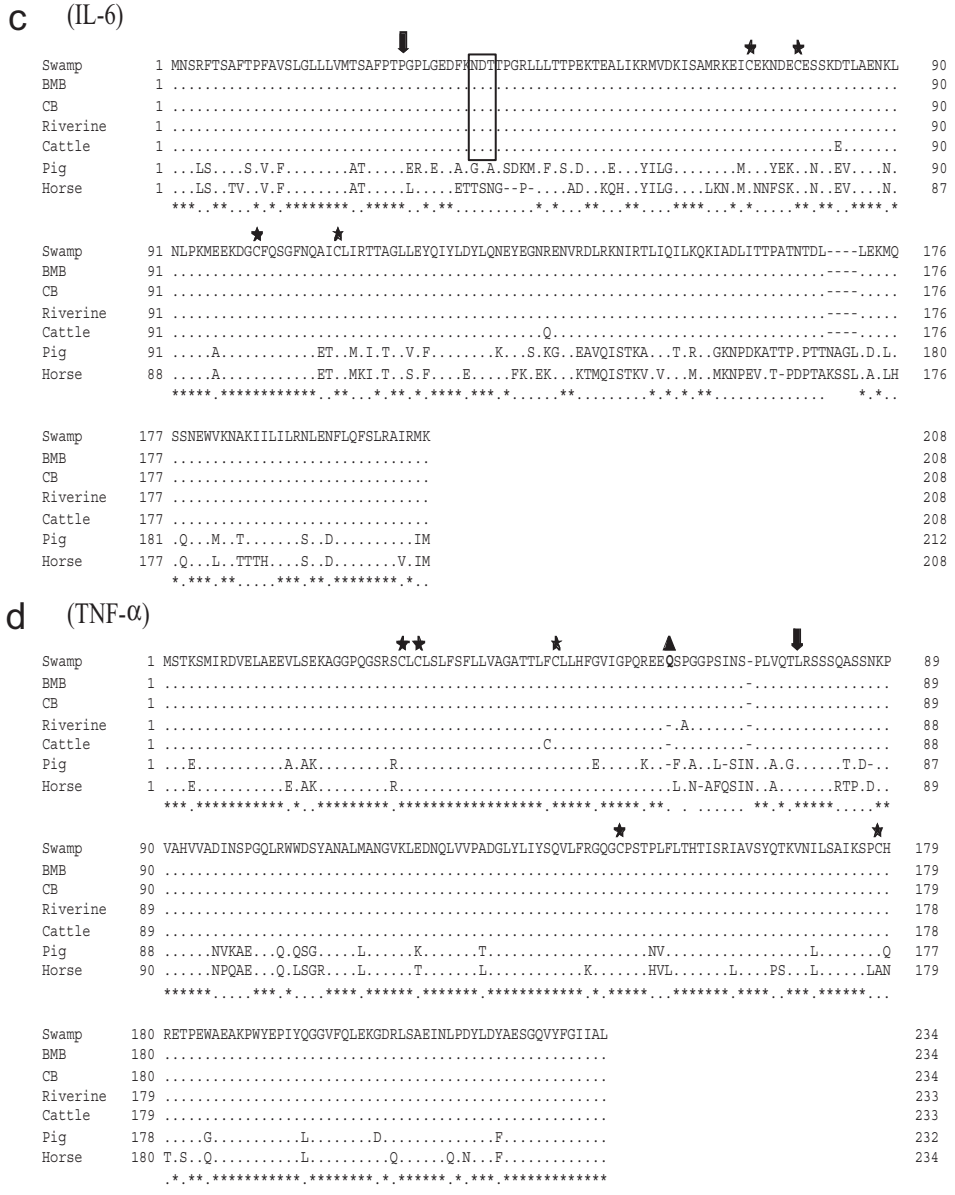

Fig. 1. (*Continued*)

Inflammatory cytokines of the tested animals showed a high degree of homology in all the compared species belong to order Artiodactyla including horse (Perissodactyla). However, homology was relatively low in the other test animals. These results confirmed a closer phylogenetic relationship between members of this

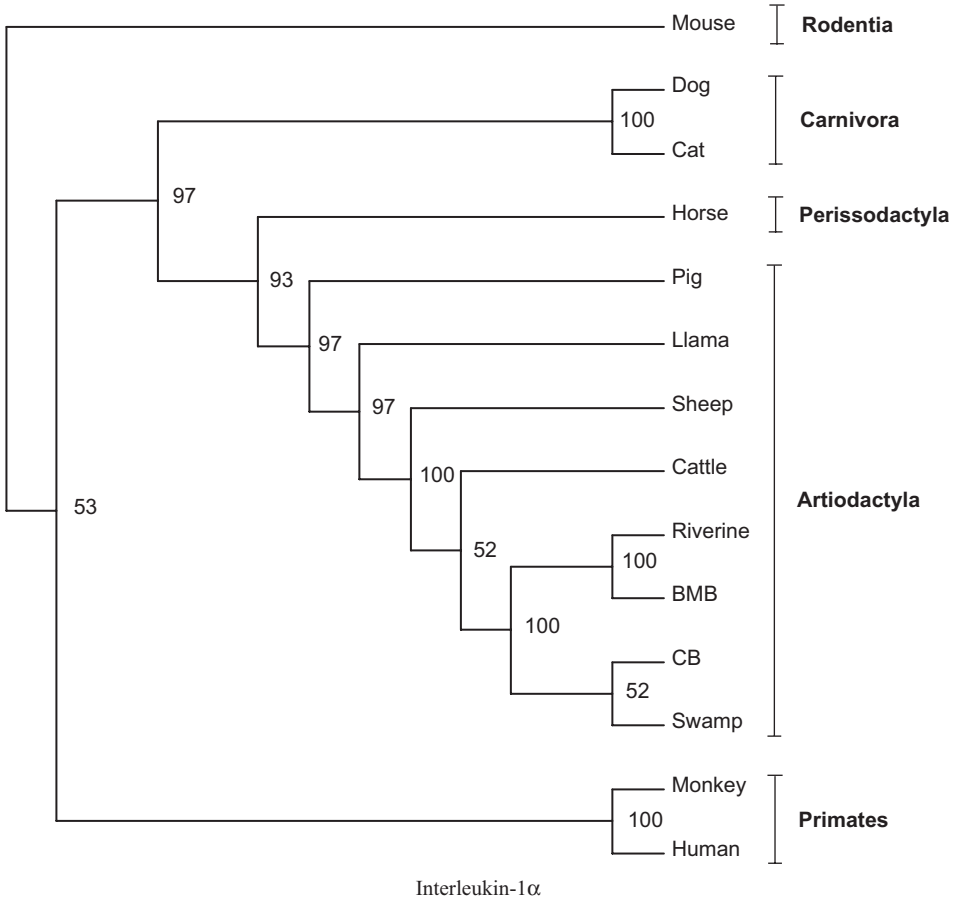

Fig. 2. Phylogenetic tree of IL-1α as representative figure of all the inflammatory cytokines. Nucleotide sequence of IL-1α from various species available in the GenBank was aligned. The bootstrap values supporting a particular cluster are shown within the node. Clusters were identified based on their order classification.

order. The potential N-linked glycosylation sites of these inflammatory cytokines were conserved within tribe Bovini although some of these sites were also similar with pig, which is a requirement for the retention of cytokine protein activities. The high homology is further ascertained by the stable Cys residues, which play a vital role in determining the tertiary structure and functional integrity of the protein [22]. These could also suggest that these cytokines have biological properties similar with other species.

The conserveness between species of IL-1α and IL-1β was further confirmed by the presence of carboxyl-terminal endpoint for active proteins (TDF), which was found at the same site. This may be required for the retention of cytokine protein activities in mediating immune response [23].

Furthermore, the reserveness of the IL-6 sequence, which was confirmed by the presence of four invariant cysteines is probably involved in maintaining the overall structure of the protein. Since this cytokine is a B cell growth and differentiation factor, it could possibility act as an autocrine factor for tumor cell growth [24]. Also, it has an important role in maintaining homeotasis through differentiation and growth-promoting effects on a variety of target cell types, and in releasing the effect of inflammatory response [25].

Meanwhile, although TNF-α has a central role in the host inflammatory response against viral, bacterial and parasitic infections [26], it could also be both beneficial [27] and detrimental to the host [28]. Significantly, almost similar to the previous work on cloning and sequencing of IL-10 [19], an additional amino acid, glutamine, was found in all of the TNF-α sequences of the three newly subjected bubaline breeds, which was at position 63 and supposedly between position 62 and 63 of the existing sequences at the GenBank. Previous study [29] proves that glutamine is a precursor amino acid of pyrrolid-2-one-5-carboxylic acid at the N-termini of immunoglobulin mature L-chains. This may also indicate that glutamine is an important component of TNF-α immune response.

The result of the phylogenetic analysis showed further confirmation of the relationship within the order Artiodactyla where the bubaline species, cattle and pig belong to. The bootstrap value (%) further strengthens the close relationship between the compared bubaline species and other species within the order Artiodactyla. Also, it illustrates the degree of heterogeneity of these individual species with each other.

Although this study showed that both riverine and swamp-type buffaloes including other breeds had high homology, a comparative in-depth study of their cytokine profile and expression is suggested to elucidate the differences concerning immunological reactions and resolve those claims that the latter is more stable and disease-resistant than the former [20,21].

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