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MOLECULAR ANALYSIS OF SOLUTE CARRIER FAMILY 11 MEMBER A1 (SLC11A1) GENE IN RUMINANTS AND NON-RUMINANTS USING COMPUTATIONAL METHOD

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Solute carrier family 11 member a1 gene (Slc11a1) previously known as natural resistance-associated macrophage protein 1 (Nramp1) is a gene of member of family of metal ion-transport protein. The cellular expression is restricted to phagocytic cells. Slc11a1 delivers bivalent metal cations from the cytosol into acidic endosomal and lysosomal compartments where Fenon and Haber-Weiss reaction generates toxic antimicrobial radicals for direct antimicrobial activity against harmful microorganisms. The present study was undertaken with the objective of analyzing Slc11a1 gene to gain insight into the evolutionary proximity and divergence as well as the polymorphism of this gene in ruminants and non-ruminants including the attendant effects of the genetic variants on the function of the Slc11a1 protein. Thirty Slc11a1 gene sequences of 6 mammalian species classified as ruminant (goat, sheep, cattle, *Bubalus bubalis* and *Bubalus carabanensis*) and non-ruminant (swine and horse) animals were investigated. The length of the Slc11a1 gene varied from 448-2,357. There was substantial genetic variation and polymorphism in the aligned sequences of Slc11a1 gene within and across species. Functional analysis of non-synonymous mutations in cattle revealed that twenty five of the amino acid substitutions at the peptide binding region could be beneficial, (E36G), (T52A), (N161S) and (V248I) were likely to be beneficial while only (Q312K) was harmful. In horse, two of the amino acid mutations were harmful, two were likely to be harmful, one was undecided, four were likely beneficial and the rest twenty were beneficial. The phylogenetic trees showed some form of proximity and differentiation

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in Slc11a1 sequences within and across species. The present information on the polymorphism of Slc11a1 gene might be exploited in the search for association with disease resistance in Nigerian livestock species.

Key words: functional analysis, mammals, polymorphism, phylogenetic trees.Slc11a1 gene

INTRODUCTION

The Solute Carrier family 11 member A1 (Slc11a1) gene, previously known as Natural Resistance Associated Macrophage Protein 1 *(NRAMP1)* gene is a member of large family of metal ion-transport proteins and it was the first positional cloned gene related to infectious disease susceptibility in mouse (VIDAL *et al.,* 1993). Slc11a1 was first discovered by three independent research groups after observations of animal infection models. The first group designated the locus *Lsh* after the observation that inbred strains of mice exhibited differential growth of *Leishmania donovani* within macrophages (BRADLEY, 1977). A similar observation was made following infection of inbred mice with the macrophage trophic pathogen *Salmonella typhimurium*, where the resistance locus was named *Ity* (Plant and Glynn, 1974). The third group found that strains of inbred mice separated into susceptible and resistant groups when infected with *Mycobacterium bovis* (another macrophage trophicorganism) and the disease locus was named *Bcg* (SKAMENE *et al.*, 1982). It was hypothesized that susceptibility, or resistance, to the three infectious organisms was controlled by a single locus, which encoded a protein that modulated macrophage function (Blackwell, 1989). It was further shown that the gene had restricted expression to reticuloendothelial organs; namely the spleen, liver and blood. Consequently, the gene was named natural resistance-associated macrophage protein 1 or *Nramp1* (MALO *et al.*, 1994; VIDAL *et al.*, 1993).

Using positional cloning to determine the location of the *Bcg*/*Lsh*/*Ity* locus on mouse Chromosome 1, it was discovered that susceptibility to macrophage trophic pathogens was the result of a point mutation in the coding region of *Nramp1* (VIDAL *et al.*, 1993). This mutation, leading to a single non-conservative amino acid substitution of a glycine for an aspartic acid residue at position 169 (G169D) in trans-membrane domain 4, produces a non-functional protein (VIDAL *et al.*, 1996). Sequence analysis of *Nramp1* from 27 inbred mouse strains found concordance between the presence of the wild type G or mutant D amino acid at position 169 and resistance or susceptibility to infection, respectively (MALO *et al.*, 1994).

Since Slc11a1 is associated with pH dependent transport of divalent cations (like Fe2+, Mg2+ etc) through the phagosome membranes, they are essential for many cellular functions. Experimental studies suggest that the transport is from the lumen of the phagolysosome to the cytosol, which prevents the acquisition of these cations by intracellular pathogens (FORBES and GROS, 2003*)*. Under normal physiological conditions, Slc11a1 gene delivers bivalent metal cations from the cytosol into acidic endosomal and lysosomal compartments where the Fenton and Haber-Weiss reaction generate toxic antimicrobial radicals for direct antimicrobial activity against phagocytosed microorganisms (GOSWAMI *et al*., 2001). Slc11a1 gene have pleiotropic effects on macrophage function, that include increased chemokine KC, tumour necrosis factor-α, interleukin-1β, inducible nitric oxide synthase and major histocompatability complex class II expression; all are important in resistance to intracellular pathogens (AWOMOYI, 2007).

There are so many experimental reports regarding the association of Slc11a1 gene polymorphisms with resistance to infectious diseases in human beings (THOMAS and JOSEPH,

2012). However, in livestock, there is dearth of information on the avalanche of data available in the GenBank as regards the evolution and differentiation of the gene within and among species including the functionality of the amino acid mutations of the gene. The method to identify functional SNPs from a pool, containing both functional and neutral SNPs is challenging by experimental protocols (George *et al*., 2008). Therefore, computational predictions have become indispensable for evaluating the disease-related impact of non-synonymous single-nucleotide variants discovered in exome sequencing (LIU and KUMAR, 2013; YAKUBU, 2014). The present investigation aimed at examining the genetic diversity of Slc11a1 gene especially on its evolution and differentiation within and among species as well as the attendant effects of the polymorphism on the function of Slc11a1 gene.

MATERIALS AND METHODS

A total of thirty (30) Slc11a1 gene sequences from six species [*Capra hircus* (4), *Ovies aries* (4), *Bos taurus* (2), *Bubalus bubalis* (2), *Bubalus carabanensis* (2), *Sus scrofa* (12), and *Equus ferus caballus* (4)], were obtained from the GenBank. The GenBank accession nos. are goat (GU440577.1, JF431430.1, EU924641.1 and FJ38877.1), sheep (NM001009345.1, AF005380.1, U70255.1 and AF128882.1), cattle (NM174652.2 and BT026247.1) *Bubalus bubalis* (FJ827149.1 and FJ827151) *Bubalus carabanensis* (FJ827148.1 and FJ827150.1) swine (NM213821.1, EF200586.1, EF200585.1, AY368479.1, EF200584.1, EU135795.1, AY368478.1, AY368477.1, AY368475.1, AY368473.1, AY368472.1 and AY368471) and horse (NM001081857.1, AF163298.1, AF163297 and AF354445.1). Sequence alignments, translations and comparisons were carried out using ClustalW (LARKIN *et al*., 2007).

A neighbour-joining tree on the basis of genetic distances, depicting phylogenetic relationships among Slc11a1 nucleotide sequences of the investigated species was constructed using the complete deletion and p-distance options. The reliability of the tree was estimated by bootstrap confidence values (FELSENSTEIN, 1985), with 1000 bootstrap replications. Similarly, a consensus sequence of each of the published goat, sheep, cattle, *Bubalus bubalis, Bubalus carabanensis*, swine and horse sequences was used to obtain a phylogenetic tree using the UPGMA method of MEGA5 software (TAMURA *et al*., 2011). The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.2). All sequences were trimmed to a similar length (448bp) corresponding to the same region before generating the tree.

A functional analysis of amino acid mutations of horse and cattle was obtained using MEGA-MD (STECHER *et al*., 2013). MEGA-MD was used to analyse the effect of a change in amino acid coding SNP and a functional impact on the protein. MEGA-MD is a suite of tools developed to forecast the deleteriousness of nsSNVs using multiple methods (EvoD, PolyPhen-2 and SIFT) and to explore nsSNVs in the context of the variability permitted in the long-term evolution of the affected position (STECHER *et al*., 2013). In its graphical interface for use on desktops, it enables interactive computational diagnosis and evolutionary exploration of nsSNVs. As a web service, MEGA-MD is suitable for diagnosing variants on an exome scale. MEGA-MD automatically retrieves a 46-species protein sequence alignment that comes from the UCSC resource (Fujita *et al*., 2011), which has been cached in the MD-DB for quick access. For the selected position, there exists the option to request diagnosis for a specific variant or all possible variants (STECHER *et al*., 2013). Conclusions were drawn from the consensus which gave the average prediction between polyphen-2, SIFT and EvoD.

RESULTS AND DISCUSSION

The length of the Slc11a1 gene sequences varied from 448-2,357 within and across species (Table 1). The Solute Carrier family11 member A1 (Slc11a1) gene is a member of large family of metal ion-transport proteins and it was the first positional cloned gene related to infectious disease susceptibility in mouse (VIDAL *et al.,* 1993). The length variation of the Slc11a1 gene within and among species might result from evolution and differentiation. Many length variations caused by insertions and deletions resulting in amino acid variation within species have been found by comparison with known sequences.

Table 1. Slc11a1 gene sequence variation within and among ruminant and non-ruminants

Species	Number of sequences	Sequence length variation (bp)
Capra hircus	4	448-1998
Ovies aries	4	1494-2152
Bos taurus	2	1040-2276
Bubalus bubalis	2	1647-1723
Bubalus carabanensis	2	1647-1723
Sus scrofa	12	455-2357
Equus ferus caballus	4	533-1877

bp=base pairs

Twenty nine amino acid mutations of Slc11a1 gene sequences were analyzed in horse (Table 2). It was found that L201C and A339L were deleterious; A223P and L327H were likely deleterious, V132L was found to be ambiguous; E36G, K313Q, H328Q and M336R were likely neutral while the rest twenty were neutral. Similarly, thirty amino acid mutations of Slc11a1 gene sequences were analyzed in cattle (Table 3). The substitution Q312K was found to be likely deleterious, E36G, T52A, N161S and V248I were likely neural while the rest twenty five amino acid substitutions were neutral. Gene polymorphism between and among species appeared to be diverse in the present study. Single Nucleotide Polymorphisms (SNPs) are being intensively studied to understand the biological basis of complex traits and diseases (GEORGE *et al*. 2008). A study conducted by RUIZ-LARRANAGA *et al.* (2010) suggested that SNP c.1067C > G of Slc11a1 gene may be a potential causal variant that causes an amino acid change in codon 356 from proline to alanine (P356A) that could alter Slc11a1 protein function; although functional studies are needed to assure this point and this association study supports the involvement of Slc11a1 gene in susceptibility to MAP infection in cattle. In some related studies, Bovine tuberculosis caused by *Mycobacterium bovis,* is a considerable health hazard to animal keepers and general communities. A microsatellite polymorphism within the Slc11a1 gene (allele 211, allele 215 and allele 217) is significantly related to lower incidence of bovine tuberculosis in Chadian cattle (African Zebu) (KADARMIDEEN *et al.,* 2011). CAPPARELLI *et al.* (2007a, 2007b) reported a significant association of polymorphisms at 3′UTR of Slc11a1 gene with resistance/susceptibility to brucellosis in buffalo (*Bubalus bubalis*). A significant association was also found between the *B. abortus* macrophage in vitro killing assay phenotypes and the bovine Slc11a1 3'UTR genotypes, which suggests that the A allele may be associated with resistance (MARTINEZ *et al.,* 2008). REDDACLIFF *et al. (*2005*)* identified Slc11a1 gene polymorphisms in two phenotypically defined Merino flocks with a high prevalence of MAP infection, and possible association with susceptibility/ resistance to Johne's disease was detected. Liandris *et al.* (2009)

sequenced the caprine Slc11a1 gene (GeneBank FJ388877) and investigated the potential association of its polymorphisms with test positivity of goats to MAP infections. In a similar study conducted in goats in Greece, it was observed that the 3'UTR of caprine Slc11a1 gene contains two microsatellites with a variable number of guanine-thymine (GT) repeats named region A and B and statistically significant association was established between genotypes of region B and ELISA (Enzyme Linked Immuno Sorbent Assay) results of paratuberculosis (KOROU *et al.,* 2010; VACCA *et al.* (2011). Therefore, the beneficial amino acid mutations in the present study when validated in functional association studies using the wet laboratory may be exploited in the selection of disease-resistant individuals as indicated in earlier studies (CHOI *et al*., 2012).

Table 2. Functional analysis of amino acid mutations in horse

Position (AA)	Reference (AA)	Mutant (AA)	Consensus	EvoD	Polyphen-2	SIFT
5	K	S	Neutral	36.35	NA	NA
7	P	A	Neutral	41.77	0.004	0.73
8	Q	P	Neutral	37.40	0.001	0.37
12	G	R	Neutral	12.40	0	0.62
13	S	P	Neutral	22.12	0.001	0.36
24	P	S	Neutral	44.50	0.002	0.41
25	T	P	Neutral	18.88	0	0.30
35	R	G	Neutral	41.92	0.001	0.38
36	E	G	Likely Neutral	31.48	0.054	0.39
49	K	E	Neutral	24.53	0.003	1.00
50	P	Q	Neutral	48.31	0.004	0.59
132	V	L	Ambiguous	59.62	0.008	NA
201	L	C	Deleterious	69.89	NA	NA
220	E	A	Neutral	33.30	0.004	0.29
223	A	P	Likely Deleterious	63.55	0.021	0.02
272	A	S	Neutral	43.21	0.006	1.00
297	I	L	Neutral	30.20	0.009	0.61
303	M	V	Neutral	40.84	0.002	1.00
313	K	Q	Likely Neutral	12.56	0.014	0.19
318	A	V	Neutral	3.662	0.002	1.00
327	L	H	Likely Deleterious	78.56	0.033	0.54
328	H	Q	Likely Neutral	54.33	0.004	0.42
336	M	R	Likely Neutral	58.02	0	0.53
339	A	L	Deleterious	58.64	NA	NA
366	I	V	Neutral	-33.27	0.019	0.93
392	R	K	Neutral	13.62	0.002	0.21
421	L	V	Neutral	27.72	0.006	1.00
454	T	A	Neutral	26.10	0	0.96
455	L	I	Neutral	43.34	0.012	1.00

AA=amino acid, M=methionine, I=isoleucine, L=leucine, V=valine, C=cysteine, A=alanine, G=glycine, P=proline, T=threonine, S=serine, W=tryptophan, Q=glutamine, N=asparagine, H=histidine, E=glutamic acid, D=aspartic acid, K=lysine, R=argin NA=not available

Table 3. Functional analysis of amino acid mutations in cattle

Position (AA)	Reference (AA)	Mutant (AA)	Consensus	EvoD	Polyphen-2	SIFT
2	T	S	Neutral	27.92	0.001	1.00
5	K	T	Neutral	32.74	0.003	0.63
8	Q	P	Neutral	37.40	0.001	0.37
9	R	K	Neutral	25.67	0.001	0.94
10	L	Q	Neutral	43.62	0.004	0.70
11	S	G	Neutral	40.95	0.002	0.39
13	S	T	Neutral	27.50	0.001	0.77
14	S	R	Neutral	45.94	0.005	0.53
25	T	P	Neutral	18.88	0.000	0.30
28	G	E	Neutral	29.57	0.003	1.00
35	R	G	Neutral	41.92	0.001	0.38
36	E	G	Likely Neutral	31.48	0.054	0.39
49	K	E	Neutral	24.53	0.003	1.00
50	P	S	Neutral	45.64	0.001	0.98
52	T	A	Likely Neutral	66.72	0.014	1.00
135	T	I	Neutral	37.42	0.002	1.00
136	V	L	Neutral	-3.27	0.007	0.50
161	N	S	Likely Neutral	45.65	0.034	0.33
178	I	V	Neutral	25.29	0.029	0.26
201	L	F	Neutral	1.92	0.004	1.00
218	R	Q	Neutral	48.92	NA	NA
220	E	A	Neutral	33.30	0.004	0.29
226	R	Q	Neutral	22.04	0.015	0.20
238	H	Q	Neutral	28.25	0.001	0.47
248	V	I	Likely Neutral	50.68	0.034	0.29
262	A	S	Neutral	11.17	0.186	0.07
269	I	V	Neutral	42.92	0.020	0.84
272	A	S	Neutral	43.21	0.006	1.00
297	I	L	Neutral	30.20	0.009	0.61
312	Q	K	Likely Deleterious	64.29	0.004	0.09

AA=amino acid, M=methionine, I=isoleucine, L=leucine, V=valine, C=cysteine, A=alanine, G=glycine, P=proline, T=threonine, S=serine, W=tryptophan, Q=glutamine, N=asparagine, H=histidine, E=glutamic acid, D=aspartic acid, K=lysine, R=arginine
NA= not available

The neighbour-joining tree clearly revealed trans-species evolution (Figure 1). From the neighbour-joining tree, it was observed that swine sequences with accession numbers; EF200584.1, EU135795.1, NM213821and AY368478.1 clustered more. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution (Baum, 2008). The presence of numerous alleles at a particular Slc11a1 gene locus is an evidence of a long-term evolutionary persistence of the locus. This is suggested by the fact that the alleles in one species are often

more closely related to the alleles in closely related species than to the other alleles in the same species ((TAKESHIMA *et al*., 2008; YAKUBU *et al*., 2013a and b).

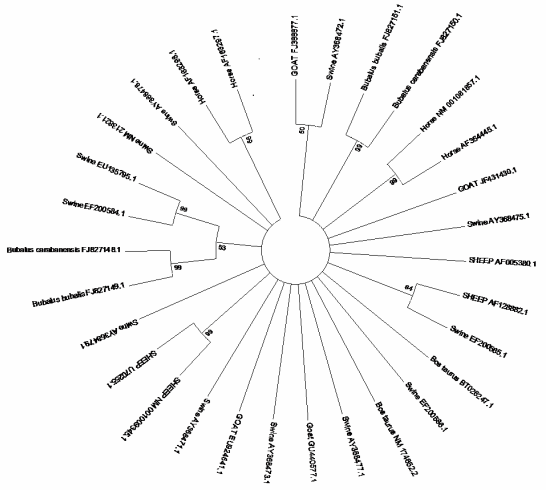

Figure 1. Neighbour-joining tree obtained from Slc11a1 gene in some ruminants and non-ruminants derived using the Neighbour-Joining method.

The average nucleotide substitutions per site (D_{xy}) between selected species nucleotide sequences from 6 mammalian species are presented in Table 4. Among the ruminants, goat and *Bubalus bubalis* as well as *Bubalus carabanensis* shared the highest D_{xy} value of 0.764. The smallest D_{xy} value of 0.730 was found between goat and sheep. In non-ruminants, the D_{xy} value found between horse and swine was 0.730. Dxy is the index of DNA divergence between or among the sequences. The larger the Dxy value, the greater the genetic distance while the smaller the Dxy value the closer the genetic distance between the species (KANG *et al*., 2008). This can be seen in the dendrogram (UPGMA) (Figure 2) drawn from the consensus sequences of the Slc11a1 gene of the ruminant and non-ruminant animals.

Table 4. The average nucleotide substitutions per site (D_{xy})

Species	Goat	Sheep	Cattle	*B. bubalis*	*B. carabanensis*	Swine	Horse
Goat		0.023	0.023	0.023	0.023	0.022	0.022
Sheep	0.730		0.022	0.022	0.022	0.023	0.024
Cattle	0.741	0.759		0.023	0.023	0.023	0.023
B. bubalis	0.764	0.759	0.730		0.000	0.022	0.024
B. carabanensis	0.764	0.759	0.730	0.000		0.022	0.024
Swine	0.744	0.727	0.753	0.756	0.756		0.023
Horse	0.787	0.736	0.736	0.710	0.710	0.730	

The number of base substitutions per site from between sequences is shown below the diagonal. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates).

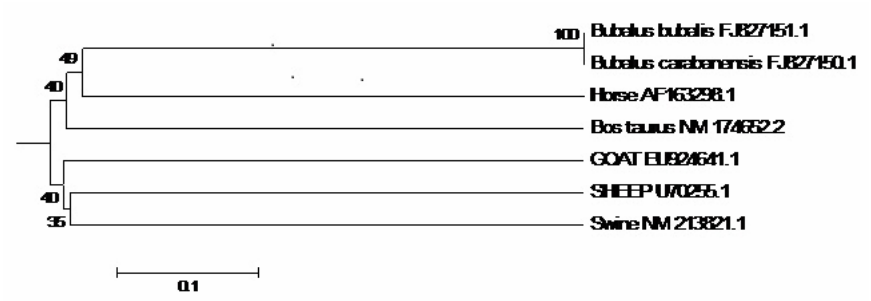

Figure 2. A Phylogenetic tree derived from consensus sequences of Slc11a1 gene of some ruminants and non-ruminants using the UPGMA method.

CONCLUSIONS

There was a great genetic variation and polymorphism in the aligned sequences of Slc11a1 gene in ruminants and non-ruminants. Computational analysis of non-synonymous mutations showed that in horse two of the amino acid mutation were harmful, two were likely to be harmful, one was undecided, four were likely beneficial and the rest twenty were beneficial. However, in cattle, only one of the amino acid substitutions was harmful and four were likely to be beneficial while twenty-five were beneficial. The result obtained revealed some form of variations leading to divergence in Slc11a1 sequences in ruminants and non- ruminants. The present information on the polymorphism of Slc11a1 gene might be exploited in search of association with disease resistance and other productive and reproductive functions in livestock species especially those found in developing countries such as Nigeria, sub-Saharan Africa.

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MOLEKULARNA ANALIZA SOLUTE PROTEINA NOSIOCA PORODICE 11 ČLANOVA a1(S1c11a) GENA KOD PREŽIVARA I NEPREŽIVARA KORISTEĆI RAČUNSKI METOD

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Izvod

Solute protein, nosioc familije 11 članova a1 gena (S1c11a1), ranije poznat kao prirodni, sa rezistentnošću povezan makrofagni protein 1(Nramp1) je gen član familije metal-jon transport poteina. Ćelijska ekspresija je ograničena na fagocitne ćelije. Slc11a1 oslobađa bivalentne katjone metala iz citozola u kiseli endosomalni i lizozomalni proctor gde Fenon i Haber-Weiss reakcija generiše toksične antimikrobne radikale za direktnu antimikrobnu aktivnost protiv štetnih mikroorganizama. Vršena su istraživanja sa ciljem objektivne analize Slc11a1 gena I uvida u evolucione srodnosti ili divergentnosti kao I polimorfizam tih gena kod preživara I nepreživara, uključujući efekat genetičkih varijanti na funkcijugain Slc11a1 proteina. Vršena su ispitivanja trideset Slc11a1 sekvenci gena kod 6 vrsta životinja klasifikovanih kao preživari (koza, ovca, krava, *Bubalus bubalis* I *Bubalus carabanensis*) i nepreživara (svinja i konj). Dužina Slc11a1 gena varira od 448 - 2,357 kb. Utvrđeno je suštinsko genetičko variranje I polimorrfizam sekvenci Slc11a1 gena unutar i između vrsta. Filogenetska stable su pokazala formu bliskosti i diferencijacije u Slc11a1 sekvencama unutar i između vrsta. Ova informacija o polimorfizmu Slc11a1 gena može da se koristi u istraživanju asocijacije sa rezistentnosti na bolesti kod vrsta domaćih životinja u Nigeriji.

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