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Analysis of *SLC11A1* gene expression in healthy water buffalo (*Bubalus bubalis*) blood cells using qPCR

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ABSTRACT. *SLC11A1* (solute carrier family 11 member 1 protein) gene influences the initial phase of bacterial cellular infections through macrophage activation. Recent literature on buffalo has attempted to associate the genotype of the polymorphic microsatellite located in the 3'untranslated region (3'UTR) of the gene, with either susceptibility to brucellosis or with improved macrophage function. Carriers of the $(GT)_{16}$ allele have been reported to be resistant to brucellosis. In this study we analyzed the steady-state level of *SLC11A1* expression in a serologically negative herd of 26 animals differing by the number of $(GT)_n$ microsatellite repeats by using a reverse transcriptase quantitative real-time polymerase chain reaction approach. We evaluated five different reference genes, which had not been reported previously, for use in gene expression experiments in buffalo blood. However, we did not find any significant difference between buffalo carriers of the different microsatellite alleles, with respect to *SLC11A1* expression in whole blood or in blood fractions [peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes/granulocytes (PMN/G)]. Conversely, there was a difference between the blood fractions in their

SLC11A1 expression levels, with the PMN/G fraction having a higher expression level than the PBMC fraction ($P < 0.015$).

Key words: *SLC11A1* gene expression; Buffalo; Blood cells; Reference gene (RG); qPCR

INTRODUCTION

Brucellosis continues to be a serious worldwide bacterial zoonosis of major significance to animal and human populations. Host genetic, innate and adaptive immune factors significantly influence the outcome of brucellosis infection, as does the strategies by which intracellular *Brucella* to evade host factors. This has resulted in a delicate co-evolutionary balance between the long-term survival of the host and the pathogen (Adams and Schutta, 2010).

The protein solute carrier family 11 member 1 (SLC11A1, formerly NRAMP1, natural-resistance-associated macrophage protein) is expressed mainly in the phagosome of immune system cells, such as macrophage and neutrophils, and determines the resistance or susceptibility of the host to intracellular pathogens by influencing the initial phase of bacterial cellular infections and regulating macrophage activation (Blackwell et al., 2000). The role of the SLC11A1 protein is to prevent intracellular bacterial growth by means of the proton/divalent cation (mainly Fe^{2+}, Zn^{2+} and Mn^{2+}) antiporter function. The protein SLC11A1 localizes to the late endosome or lysosomes of macrophages and delivers divalent cations from the cytosol to this acidic compartment; here the Fenton reaction can use ferrous iron to generate toxic hydroxyl radicals that limit pathogen growth (Blackwell et al., 2003). The *SLC11A1* gene has pleiotropic effects on macrophage function, which include increased expression of chemokine KC, tumor necrosis factor-α, interleukin-1β, inducible nitric oxide synthase, and major histocompatibility complex class II (Awomoyi, 2007).

SLC11A1 belongs to a group of genes that are highly conserved across various mammalian species (Vidal et al., 1993). The role of *SLC11A1* is very important due to its association with resistance and susceptibility to various intracellular pathogens in humans as well as in livestock species (Thomas and Joseph, 2012).

Different $(GT)_n$ microsatellite alleles, localized in the 3'UTR of the gene, have been associated with macrophage function and either susceptibility or resistance to *Brucella abortus* infections, both in cattle and in buffalo (Kumar et al., 2005; Borriello et al., 2006; Capparelli et al., 2007a,b; Martinez et al., 2008; Ganguly et al., 2008). However, contradictory results have been reported, showing that correlation with resistance to brucellosis occurs with different alleles.

De Matteis et al. (2009) sequenced the coding region and the 3'UTR of *SLC11A1* in 49 buffaloes from the same farm (23 serologically positive and 26 negative in *B. abortus* tests) and detected twelve mutations in the exons and the introns, and three allelic variants [$GT_{(9)}$, $GT_{(12)}$, and $GT_{(16)}$] of the 3'UTR microsatellite. However, they did not find any difference between serologically positive and negative animals with respect to the allele frequencies of either the exon/intron mutations or the microsatellite. In the aforementioned study, the authors noted that the $GT_{(12)}$ allele was much more frequent (67%) than the other two and that the other two existed only in a heterozygous form, which conflicts with the hypothesis that the $GT_{(16)}$ variant confers resistance to brucellosis.

The first aim of this study was to elucidate the 3'UTR of the *SLC11A1* gene in buffalo and cattle on the basis of the relevant literature. The second aim was to evaluate *SLC11A1* gene expression in a serologically negative herd of 26 animals that were polymorphic for the 3'UTR microsatellite, by using the reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR).

Data normalization is required to obtain high precision in the expression analysis of a target gene, because qPCR is subject to analytical errors that introduce variations (Bustin, 2012). This can be achieved in different ways, one of which is the use of internal controls or reference genes (RGs). The third aim of this study was also to identify an RG that could be used to normalize gene expression levels in the analysis of buffalo blood cells.

MATERIAL AND METHODS

Experimental design

Our study was performed on a buffalo herd located in the Province of Rome, Italy, where brucellosis is not endemic and included a total of 26 animals, that were serologically negative for brucellosis and carried two different *SLC11A1* 3'UTR microsatellite genotypes: group 1 with $GT_{(12)}$ homozygotes; group 2 with $GT_{(12)}/GT_{(16)}$ heterozygotes. We analyzed *SLC11A1* expression in total blood samples collected from the jugular vein of study animals. For four buffaloes of group 1 and three buffaloes of group 2, we analyzed target expression from: a) total blood, b) peripheral blood monocuclear cells (PBMC) isolated by density gradient following the manufacturer protocol (Lymphoprep™, Axis-Shield PoC AS, Sentinel CH. SpA), and c) blood depleted of PBMC (polymorphonuclear leukocytes/granulocytes, PMN/G).

Selection of genes and primer design

From the literature, we searched for a candidate RG to be tested in the buffalo blood samples; it is widely accepted that the suitability of a reference gene for any type of qPCR experiment is not given *a priori* but must be evaluated repeatedly (Hruz et al., 2011). We selected three candidate genes that had already been tested in cattle [ATP synthase beta polypeptide, nuclear gene encoding mitochondrial protein (*ATP5B*); eukaryotic translation initiation factor eIF-2B subunit beta (*EIF2B2*); succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*); http://www.primerdesign.co.uk/geNorm%20gene%20lists.html] and two genes already analyzed in various tissues and in blood cells [DNA-directed RNA polymerase fragment RNA polymerase II (*POLR2A*); TATA-Box binding protein (*TBP*)] (Radonic et al., 2004).

No buffalo sequences for the selected RG were present in GenBank, so primers were designed based on the *Bos taurus* sequences by using the QuantPrime software (Arvidsson et al., 2008) and were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Primers were designed, when possible, to amplify a region spanning the introns of their respective candidate gene. Table 1 reports information relating to the primers used to amplify the target gene and candidate RGs, and reports expected amplicon sizes. Preliminary amplifications were performed both on cDNA and genomic DNA to validate the primers. PCR products were sequenced using 3500 Genetic Analyzers (Applied Biosystems, Foster City, CA, USA). We subsequently verified amplification specificity by using BLAST (Altschul et al., 1997).

Table 1. Primer sequences, reference sequence, position and covered region on the mRNA, expected amplicon size for *SLC11A1*, and RG amplifications.

Gene symbol	Primer sequences	Ref. seq.	Position	Amplicon
SLC11A1	F: 5'-TTTGTGATGGAGGGCTTCCT-3' R: 5'-CAGCACATTGAGCAGGTCGTT-3'	U27105	Ex11-12fw (1217-1236) Ex12rw (1346-1366)	149
ATP5B	F: 5'-TTTGGACTCCACGTCTCGCATC-3' R: 5'-TCCTGGAGGGATTTGTAGTCCTG-3'	NM_175796.2	Ex8fw (1219-1240) Ex9rw (1304-1326)	108
EIF2B22	F: 5'-CCGTTCCCATTATGCTCAACTCCAG-3' R: 5'-TCCGTTGTCCCTTCCAGTTCCAC-3'	NM_001015593.1	Ex3fw (395-419) Ex3/4rw (453-475)	81
SDHA	F: 5'-ACGATTACTCCAAGCCCATCCAG-3' R: 5'-AACGTAGGAGAGCGTGTGCTTC-3'	NM_174178.2	Ex14fw (1825-1847) Ex14rw (1883-1904)	80
POLR2A	F: 5'-ATTGCCGCCAACATGACCTTTG-3' R: 5'-CTGCGCACTAGTTCCTGAAGTCTG-3'	XM_876181.2	Ex7fw (1541-1561) Ex7-8 rw (1590-1613)	74
TBP	F: 5'-TGCTTACCCACCAACAGTTCAG-3' R: 5'-TTTCTGCTCTGACTTTAGCACCTG-3'	NM_001075742.1	Ex 7 Fw (1139-1160) Ex 9 Rw (1256-1279)	141

RNA extraction and RT-qPCR

Blood samples were processed immediately after collection and total RNA was extracted by using a PerfectPure RNA Blood Kit (5 Prime, Eppendorf) following the manufacturer protocol. RNA extracts were DNA-digested using an RNase-Free DNase Set (Qiagen) and was then purified with the RNeasy MinElute Cleanup Kit (Qiagen). The total RNA concentration, quality and integrity were assessed with both fluorimetric analysis (Ribogreen, Molecular Probe) and microfluidic analysis (Bioanalyzer, Agilent). All the RNA samples used in this study were of good quality after the PBMC and PMN/G isolation procedure (RIN values between 7.5 and 9.5).

cDNA was synthesized from 1 µg RNA using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), containing both random hexamer and oligo-dT primers. Reactions were performed in a total volume of 20 µL for 30 min at 42°C; samples were stored at -20°C. Preliminary qPCR experiments were performed to optimize the annealing temperature and primer concentration both for the target gene *SLC11A1* and for the five candidate RGs.

The efficiency of PCR amplification was calculated for each gene, using a five-point standard curve (1:5 dilution per point) made using a single sample as a calibrator. qPCR was performed with a reaction volume of 20 µL using 10 ng cDNA and a Power SYBR® Green PCR Master Mix (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems). Each animal was analyzed in triplicate. Reverse transcript minus and no template controls were included. The amplification protocol was composed of a denaturation step at 95° for 10 min; 40 cycles of 95°C for 30 s, 61°C for 1 min; followed by a melting curve to assess amplicon specificity.

Data analysis and statistics

The *SLC11A1* expression analyses were performed by using the qBase^{PLUS} software (www.biogazelle.com) based on the $2^{-\Delta\Delta Ct}$ method with implementations (Hellemans et al., 2007). Relative quantity values represent the n-fold normalized expression relative to inter-run calibrators run across all plates. geNormplus analysis was performed in qBase^{PLUS} and assessed to screen the optimal set of reference genes from the panel of candidates (Vandesompele et al., 2002).

The significance of the variation in *SLC11A1* gene expression between the genotypes and blood fractions was tested by using the qBase^{PLUS} statistical Mann Whitney and ANOVA tests, and

performed on the calibrated and normalized log transformed relative quantity values.

RESULTS

To facilitate a comparison between the different reports of the *SLC11A1* gene 3'UTR we constructed a resumptive table (Table 2) and a descriptive figure (Figure 1). The considered region encompasses three microsatellite blocks, two of which are characterized by intra-species $(GT)_n$ variation (highlighted); the third block (underlined) does not differ between species of cattle and buffalo.

Table 2. 3'-UTR polymorphic region of *SLC11A1* gene.

Reference	Species	Position on GenBank	Accession	Repeats in stretch I	Repeats in stretch II
Horin et al., 1999	*Bos taurus*	1781-1804 on U12862	AF064488 AF064489	$(GT)_{12}$ $(GT)_{10}$	
Kumar et al., 2005	*Bos indicus*	1814-1988 on U12862			$(GT)_{13}$
Paixão et al., 2006, 2007	*Bos taurus* *Bos indicus*	1814-1988 on U12862			$(GT)_{13}$ $(GT)_{14}$ $(GT)_{15}$
Gonzales et al., 2006	*Bos taurus*	1814-1988 on U12862			$(GT)_{13}$
Martinez et al., 2008	*Bos taurus*	1781-1804 on U1286		$(GT)_{12}$ $(GT)_{10}$	
Capparelli et al., 2007a	*Bubalus bubalis*	1745-1955 on U27105	DQ095780 DQ095781 DQ0376109 DQ376110	$(GT)_{12}$ $(GT)_{16}$ $(GT)_{9}$ $(GT)_{12}$	$(GT)_{15}$ $(GT)_{14}$ $(GT)_{16}$ $(GT)_{16}$
Gangyly et al., 2008	*Bubalus bubalis*	1804-1996 on U27105	DQ645387 DQ645389 DQ645388		$(GT)_{13}$ $(GT)_{14}$ $(GT)_{15}$ $(GT)_{16}$

Position on GenBank and accession (if present) refers to the regions studied by the authors, repeats in stretch indicates the $(GT)_n$ variant present in the corresponding stretch as indicated in the Figure 1.

Alleles $(GT)_{16}$ and $(GT)_{12}$ are present in the first block and correspond to the alleles reported by Capparelli et al. (2007a) as B (DQ095781) and A (DQ095780), respectively. The second block present $(GT)_{14}$ and $(GT)_{15}$ repeats, respectively.

All the RG loci designed based on the *B. taurus* sequence were expressed in buffalo blood. These primers were highly specific; this was demonstrated by the single band pattern of the PCR products when run on agarose gels, and by the presence of a single peak in the dissociation curve of the qPCR. The size of the amplicons was as expected (Table 1) and the new sequences were submitted to GenBank with the following accession numbers: HM748857 (*ATP5B*), HM748858 (*EIF2B22*), HM748859 (*POLR2A*), HM748860 (*SDHA*), and HM748861 (*TBP*).

In Table 3, we reported the optimal primer concentration (nM) and temperature used in the qPCR with the slope obtained, Y intercept, R^2, and amplification efficiency of *SLC11A1* and the candidate RGs. A preliminary geNorm analysis of six samples with the five candidate RGs showed that all were very stably expressed (data not shown), so we normalized target expression on *EIF2B2*.

The analysis of *SLC11A1* gene expression in the two genotypic groups is shown in Figure 2. There was no difference between the homozygous and heterozygous animals (Mann Whitney, $P < 0.061$).

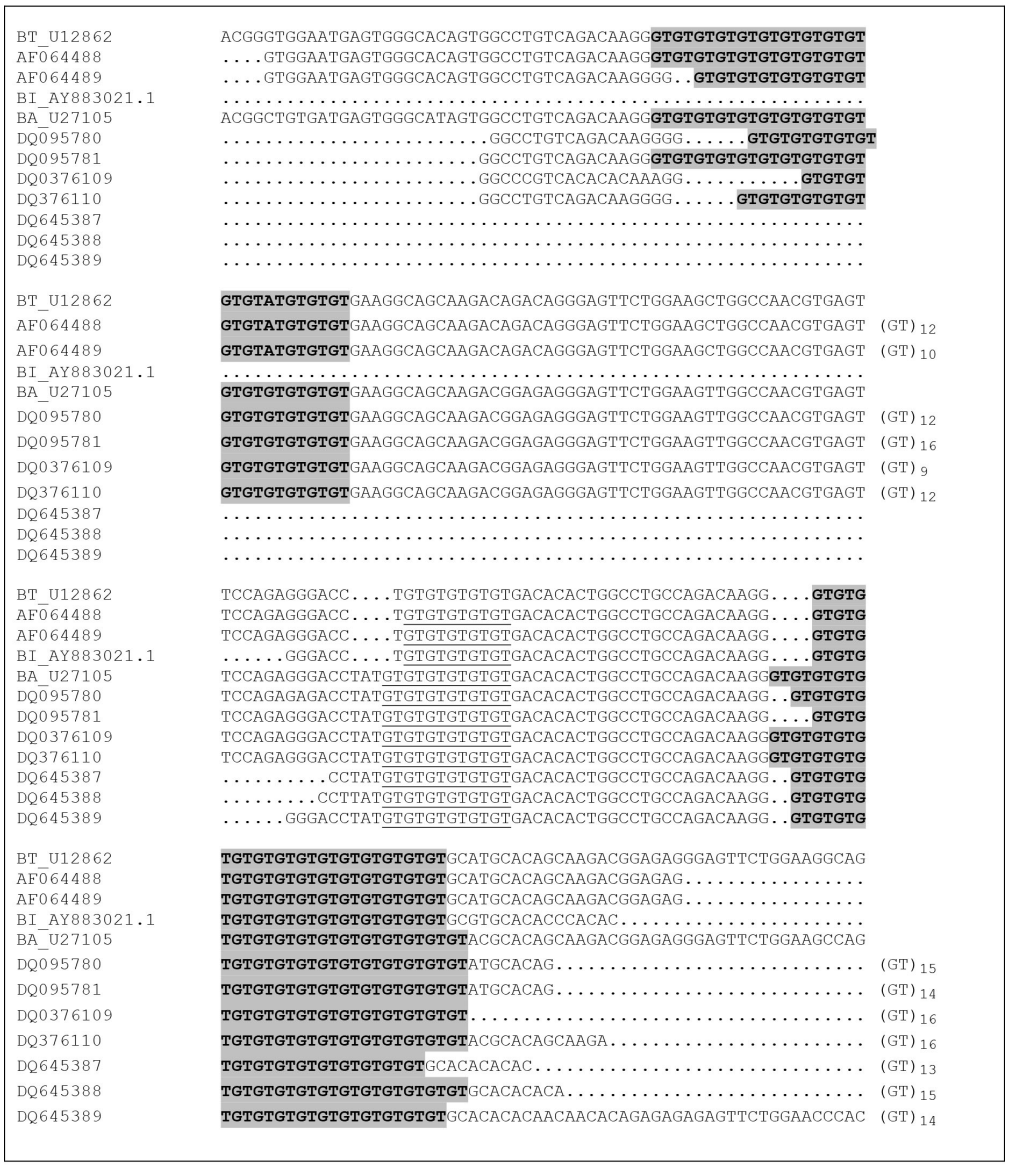

Figure 1. Alignment of bovine and buffalo *SLC11A1* 3'-UTR sequences. BT= *Bos taurus*, BI = *Bos indicus*, BA = *Bubalus arnee*.

In a second experiment, we decided to compare the *SLC11A1* expression in different blood cell fractions of four homozygous and three heterozygous samples. In Figure 3, we report the relative quantity values in the whole blood and in the PBMC and PMN/G fractions. An ANOVA showed that the cells from PMN/G fraction expressed the *SLC11A1* gene at higher level than the PBMC ($P < 0.015$). However, no difference was found between the two genotypic groups in the different fractions ($P < 0.60$).

Table 3. Results of qPCR optimization experiments.

Gene name	Primer (nM)	Slope	Yint	R^2	Eff. %	T.a.
SLC11A1	200/200	-3.191	23.96	0.998	105	61
ATP5B	200/200	-3.273	21.98	0.999	102	61
EIF2B22	200/100	-3.257	25.80	0.992	102	61
POLR2A	100/100	-3.26	24.49	0.989	102	61
SDHA	200/100	-3.20	21.78	0.998	105	61
TBP	50/50	-3.29	25.68	0.998	100	61

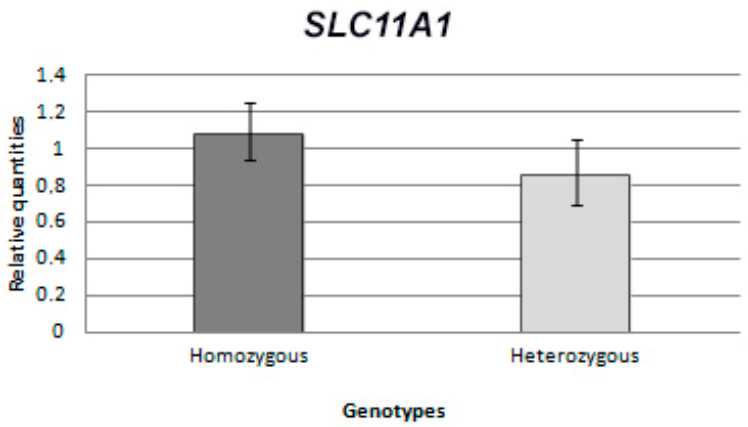

Figure 2. Relative quantities of *SLC11A1* expression in the homozygous and heterozygous groups (blood). Data reported as means with accompanying lower and upper values of the 95% confidence interval.

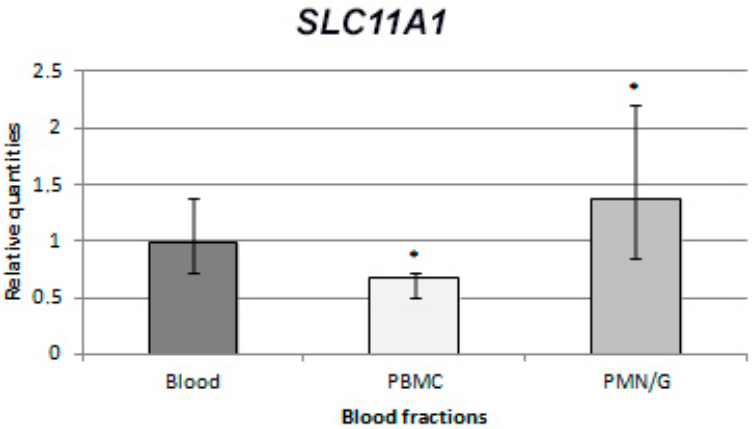

Figure 3. Relative quantities of *SLC11A1* expression in the blood and cellular fractions of 7 buffaloes. Data are reported as means with accompanying lower and upper values of the 95% confidence interval. Starred bars refer to statistical expression differences between blood fraction at $P < 0.05$.

DISCUSSION

The *SLC11A1* gene has a critical role in the innate defense against intracellular infections, and different regions of the gene have been associated with the resistance or susceptibility to various disease, both in humans and in other animals (Liu et al., 2003; Reddacliff et al., 2005; Awomoyi, 2007; Paixão et al., 2007; Martinez et al., 2008; Liandris et al., 2009; Ruiz-Larranaga et al., 2010; Li et al., 2011; Kadarmideen et al., 2011; Kumar et al., 2011; Vacca et al., 2011).

Recent literature on water buffalo (*Bubalus bubalis*) has attempted to associate the genotypes at the polymorphic 3'UTR microsatellite with either susceptibility to brucellosis or with improved macrophage function. However, contradictory results were reported (Borriello et al., 2006; Ganguly et al., 2008). In the Mediterranean buffalo there is some evidence that the $(GT)_{16}$ allele confers resistance to the intracellular pathogen *B. abortus*, in contrast with the $(GT)_{12}$ allele (Borriello et al., 2006; Capparelli et al., 2007a,b). These studies compared the *SLC11A1* expression levels in infected and non-infected monocytes from buffalo carrying different 3'UTR alleles, after *in vitro* infection assay. The highest gene expression level was recorded in the infected carriers of the $(GT)_{16}$ allele. In another study, Capparelli et al. (2007a) confirmed that non-infected $(GT)_{16}$ monocytes exhibited a basal level of *SLC11A1* expression that was approximately five times higher than those of non-infected $(GT)_{12}$ monocytes. Moreover, both infected and non-infected $(GT)_{16}$ monocytes exhibited significantly higher reactive oxygen intermediate generation than did monocytes from carriers of other alleles.

We analyzed the steady-state level of *SLC11A1* expression in the total blood and blood cellular fractions of healthy animals. Therefore, we determined qPCR protocols for both the target gene and five candidate RGs, each belonging to a different functional class, for use in blood buffalo samples.

As for the qPCR analyses, central to the concept of using an RG for normalization is the notion that expression of an RG should not vary between the tissues or cells under investigation, or in response to experimental treatments. However, no single gene is expressed at such a constant level in all such situations (Vandesompele et al., 2002). For example, *ACTB*, *GAPD*, *18S*, and *28S* rRNA are the most commonly used RGs, but a number of studies have shown that their transcription levels vary significantly between different individuals, different cell types, different developmental stages, and different experimental conditions (Radonic et al., 2004). Therefore, thorough validation of candidate RGs is critical for accurate analysis of gene expression.

In this study, we analyzed *SLC11A1* expression in blood cells of healthy animals reared in the same environmental condition. Thus, it did not matter which RG was employed; we decided to use *EIF2B2* for data normalization. Furthermore, we used RNA of optimal quality as the starting point in this study, because degradation differentially alters the stability of RGs and the q-PCR performance (Fleige et al., 2006; Fleige and Pfaffl, 2006). In all experiments reported, we corrected for efficiency; we ran inter-run-calibrations (Hellemans et al., 2007); we followed the MIQE guidelines for qPCR, when possible (Bustin et al., 2009, 2010) and performed quality control as described by Derveaux et al. (2010).

The analysis of *SLC11A1* expression in healthy buffalo showed that individuals vary greatly in their transcription levels of this gene; this variation in expression did not depend on the 3'UTR variant, in neither the total blood nor the PBMC fraction. The frequency of the $(GT)_{16}$ allele in our herd (16.6%) is lower than that reported by both Ganguly et al. (2008) and Borriello

et al. (2006). Furthermore, in a previous study of a different herd that was reared in the south of Italy, we found a similarly low frequency (18%) of this allele (De Matteis et al., 2009).

Interestingly, we found that *SLC11A1* expression was higher in the PMN/G fraction than in the PBMC fraction. While the function and expression of *SLC11A1* has been widely demonstrated in monocytes/macrophage and macrophage cell lines in different animal species, little is known about its expression in PMN leukocytes. To our knowledge only results in humans have been reported until now (Canonne-Hergaux et al., 2002).

Both PMN leukocytes and macrophages are capable of engulfing and destroying microorganisms by the action of toxic radicals, ions, and proteolitic enzymes, which are produced by these cells. Neutrophils contain different types of granules, which can be recruited for release by exocytosis or fusion with phagosomes containing ingested particles (Smolen and Boxer, 2001). Cellier et al. (1997) observed that PMN/G leukocytes are the major site of *SLC11A1* expression, followed to a lesser degree by monocytes. Canonne-Hergaux et al. (2002) detected *SLC11A1* mRNA expression in circulating PMN leukocytes and also in the most mature neutrophil precursors of healthy humans; SLC11A1 was found to be associated with the membrane granules of neutrophils, which is consistent with SLC11A1 being an integral membrane protein. The recruitment of tertiary granules to phagosomes that are formed in neutrophils after ingestion of microbial pathogens, would be expected to deliver the SLC11A1 protein to these phagosome membranes. This research suggests that SLC11A1 may have the same biochemical activity and play the same functional role in neutrophils and macrophages. Furthermore, Fierer (2001) reported that mouse PMN leukocytes, part of the innate immune system, kill *Salmonella* in a complement-dependent manner, and work in concert with macrophages.

The *SLC11A1* expression pattern that we found in the PMN/G fraction of buffalo blood resembles the human expression pattern; thus we can speculate about its role in this cell type of the innate immune system. Given the discordant results obtained between groups in this study of the allelic variants of the *SLC11A1* 3'UTR, it is simplistic to explain the complexity of the resistance/susceptibility to disease through the expression of a single gene or the production of single antimicrobial factor. It is clear that the application of tools from biology systems to the study of the innate immune system will enable comprehensive analysis of the complex interactions that maintain the fine balance between host defense and inflammatory disease (Zak and Aderem, 2009).

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