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ORIGINAL ARTICLE

Modulation of immunity and inflammatory gene expression in buffalo (*Bubalus bubalis*) with some digestive disorders

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Abstract So far, there has been scarce information about the status of immunoglobulins (Ig) and the gene expression of inflammatory cytokines in buffaloes showing digestive troubles. The purpose of the present study was to explore the modulation of gene expression of some immune-inflammatory markers in buffaloes suffered from various digestive disorders. For this reason, 50 native breed water buffaloes were studied. Forty of these buffaloes showed various symptoms of digestive disorders and were allocated into 4 groups of equal sizes (group 1: uncategorized stomatitis; group 2: acute traumatic reticuloperitonitis [TRR]; group 3: acute rumen impaction; and group 4: undifferentiated enteritis). Ten apparently healthy buffaloes were randomly selected and considered as a control group. RNA was firstly extracted from the whole blood then a reverse transcription kits was used to convert the RNA to cDNA. Real-time PCR was used to measure the expression of mRNAs of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α, IgG, and IgA, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal reference. The results of real-time PCR revealed a significant ($P \leq 0.05$) upregulation of the gene expression of IL-1β, IL-6, IL-10, and TNF-α in blood of diseased buffaloes compared with those of controls**.** Animals showing acute TRP had peak values of both IL-6 and IL-10; while those exhibiting enteritis and rumen impaction had the highest values of IL-1β and TNF-α, respectively. The results of qPCR also revealed a significant ($P \leq 0.05$) downregulation of both IgG and IgA gene expression in blood of all diseased buffaloes compared with controls**.** The lowest values of both genes were recorded in buffaloes showing acute TRP. The results herein suggest that the tested genes could have a pivotal role in the pathophysiologic mechanism of the underlying diseases.

Keywords Buffaloes · Digestive disorders · Gene expression · Cytokines · Immunoglobulins

Introduction

The water buffalo (*Bubalus bubalis*) *i*s an important species of dairy animal, particularly in countries with agriculture-based economies. The interest in water buffalo breeding has been increased significantly over the last two decades. They contribute efficiently in dairy and meat production as well as providing a great source of horns, skin besides the draught work of small farmers (Michelizzi et al. 2010).

Disorders of the digestive system provoke a great effect on the general health of affected animals. Besides, the dysfunction of the alimentary tract can impair digestion and increase susceptibility of animals to various digestive and immunometabolic alterations (Radostits et al. 2007; Youssef et al. 2017). Among the few reported systemic consequences of digestive troubles, acute phase response (APR) still receiving a few attention in buffalo species (Youssef et al. 2017). The APR, which is a component of the innate immune response, is initiated when animals are subjected to various internal or external challenges, which release cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α) (Johnson 1997). These pro-inflammatory cytokines

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induce local and systemic reactions such as pyrexia, leukocytosis, hormonal variations, and muscle protein depletion which stimulate the hepatic release of acute phase proteins (APPs) (Yoshioka et al. 2002).

Currently, there has been great interest on the usage of APPs as a marker of animal health or, alternatively, as an indicator of disease severity in veterinary medicine. It has been suggested that APPs could provide valuable diagnostic and prognostic information as well as detection and monitoring of diseases in several animal species (Ulutas et al. 2011). However, Sorensen et al. (2006) recommended using coupling of two or three APPs to help improve the diagnosis of infection or inflammation.

Although there have been several ailments affecting Buffalo species, reports about the digestive disorders and their influence on the immune system received little attention (Ibrahim 2012; Ghanem et al. 2012; El-Ashker et al. 2012, 2013, 2014; Mohan et al. 2015; Youssef et al. 2017). Up to now, there have been several studies that described the cytokine profile and the inflammatory response in buffaloes suffering from rumen drinking (El-Ashker et al. 2012), traumatic reticuloperitonitis (TRP) (El-Ashker et al. 2013, 2014), or bronchopneumonia (El-Bahr and El-Deeb 2013). In those literatures, the authors have measured the circulating levels of some inflammatory markers by using ELISA kits. However, to the best of the author's knowledge, there has been little information about gene expression of inflammatory cytokines in buffaloes showing some digestive disorders particularly stomatitis, acute TRP, acute rumen impaction, and enteritis. In addition, the influence of those disorders on the status of immunoglobulin (Ig) has not yet been studied. The objective of the present study was to explore the modulation of the gene expression of some immune-inflammatory markers including IL-1β, IL-6, IL-10, TNF-α, IgG, and IgA in buffaloes demonstrating the aforementioned digestive disorders. Our hypothesis was that these disorders would have significant inflammatory reactions or could alter the immune status of diseased buffaloes.

Materials and methods

Animal population

A total of 50 native breed water buffaloes (*B. bubalis*), with a range of 3–5 years of age and 350–500 kg body weight (BW), were studied. The studied animals were raised at different farms in Dakahlia governorate in the period between October 2015 and April 2016. Forty of these buffaloes showed various clinical symptoms including decreased appetite, signs of dehydration, teeth grinding, lowered milk yield, weight loss, lacrimation, and grunting with arched back. Some buffaloes exhibited obvious abdominal distention, and voluminous diarrhea, while others demonstrated pyrexia, profuse watery diarrhea and straining. All animals were thoroughly examined and the clinical observations were recorded. Based on the competent case history and the associating clinical findings, the investigated animals were categorized into four groups of equal sizes including uncategorized stomatitis, acute TRP, acute rumen impaction, and undifferentiated enteritis. Ten apparently healthy buffaloes, within the same range of age and BW, were randomly selected from the same farms and were considered as a control group. Animals included in this study had solely digestive disorder with no concurrent diseases and being enrolled with owner's consent and were given information about the economic impact and the potential clinical consequences of the studied ailments.

Sampling and samples processing

Ten milliliters of blood was collected from each animal via jugular vein puncture to a tube containing EDTA for adopting PCR assays. RNA was first extracted from the whole blood using a commercial RiboPure™-Blood Kit supplied by Thermo Scientific, Life Technologies (Cat No: #AM1928) according to the standard technique provided by the suppliers. A reverse transcription kits was used to convert the RNA to complementary DNA (cDNA) using a commercial kit supplied by Thermo Scientific, Fermentas (Cat No: #EP0451) according to the standard technique provided by the suppliers. For very pure samples, the absorption of ultraviolet (UV) light was used to measure the conentration of nucleic acids by using the ring structure of purines and pyrimidines. All necessary measurements and calculations were performed by using the Q5000 (UV-Vis spectrophotometer Q5000/USA).

Real-time PCR

Real-time PCR with SYBR Green was used to measure expression of messenger RNAs (mRNAs) of target genes in blood, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The isolated cDNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer's protocol (Thermo Scientific, USA, # K0221) and gene specific primers. The primers used in the amplification are shown in Table 1. The web-based tool primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design these primers based on published buffalo and cattle sequences. To ensure that primer sequence is unique for the template sequence, we checked similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 1 Forward and reverse primers sequence for the candidate genes

Gene	Forward primer (′5—′3)	Reverse primer (′5—′3)	Accession number
IL-1β	GCTTCAGGCAGGTGGTGTCGG	GCACGGGTGCGTCACACAGA	NM_001290898
IL-6	CTG CAA TGA GAA AGG AGA TA	GGT AGT CCA GGT ATA TCT GA	NM_001290980.1
IL-10	TGCCACAGGCTGAGAACCA	TCTCCCCCAGCGAGTTCA	AB246351
TNF-α	ACTCATATGCCAATGCCCTC ATG	GCAGGCACCACCAGCT	KX029323
IgG	GCAACGGAGGACTCGGCCAC	CCAGCGTCGGAGGCATCAGC	KC471582
IgA	GCTTCTTCCCGTCGGCACCC	GGCCACGGTCTTGCTGGCTT	AF109167
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	DQ882684

Relative expressions

The polymerase chain reaction mixture was carried out in a 25 μl solution which contains the following: 3 μl cDNA template (10–20 ng/ μl), 12.5 μl 2X Maxima SYBR Green/ROX qPCR Master Mix, 1 μl primer forward (10 μM), 1 μl primer reverse (0.1–0.5 μM), and 7.5 μl water, nuclease free. The final reaction mixture was placed in a StepOnePlus real-time thermal cycler (Applied Biosystems, Life technology, USA) and the PCR program was carried out with the PCR conditions in 40 cycles as follows: initial denaturation at 94 °C/10 min, denaturation at 94 °C/15 s, annealing/extension at 57 °C for all genes except IL-10 which adjusted at 62 °C/1 min. At the end of the last cycle, the temperature was increased from 60 to 95 °C to produce a melt curve.

Statistical analysis

The house keeping gene (*GAPDH*) represented as normalize that used to calculate the relative gene expression or fold change in target gene. Therefore, the quantities' critical thresholds (Ct) of a target gene were normalized with the Ct of *GAPDH* by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) as follows: the control group was used as calibrator, while other groups represented as test groups in both target and reference gene. The threshold cycle numbers (Ct) of target gene were normalized to that of reference (ref.) gene, in both the test groups and the control group by using the following equations: ΔCt (test) = Ct (target in test groups) − Ct (ref. in test groups ΔCt (calibrator) = Ct (target in control) − Ct (ref. in control). The ΔCt of the test genes were normalized to the ΔCt of the calibrator: ΔΔCt = ΔCt (test) − ΔCt (calibrator). Fold change of relative gene expression was calculated as follows: fold change = $(2^{-\Delta\Delta Ct})$.

Results

The quality and concentration of total RNA revealed the presence of pure RNA with a considerable high concentration that ranged between 900 and 1500 ng/μl. The isolated total RNA was revised transcribed into cDNA which was used as a template for qPCR. The housekeeping gene encoding *GAPDH* was used throughout the whole real-time PCR experiment as an internal reference for normalization, and the obtained data were expressed as mean ± standard error of means (SEM). The expression level of the target genes in normal buffaloes was considered the baseline. The results of real-time PCR revealed a significant ($P \leq 0.05$) upregulation of the gene expression of IL-1β, IL-6, IL-10, and TNF-α in blood of diseased buffaloes compared with those of controls (Fig. 1). Buffaloes showing acute TRP had peak values of both IL-6 and IL-10 while those exhibiting enteritis and rumen impaction had the highest values of IL-1β and TNF-α, respectively. The results of qPCR also revealed a significant ($P \leq 0.05$) downregulation of both IgG and IgA gene expression in blood of all diseased buffalo compared with controls**.** The lowest values of both genes were recorded in buffaloes showing acute TRP (Fig. 2).

Discussion

The present study set out a preliminary insight into the targeting of gene expression of some acute phase cytokines as well as IgG and IgA in buffaloes showing some digestive disorders. Previous reports have described the cytokine profile, as well as the inflammatory reaction in buffalo showing several clinical entities (Eckersall et al. 2001; Nazifi et al. 2008; El-Ashker et al. 2012, 2013; El-Bahr and El-Deeb 2013; Youssef et al. 2017). In the current investigation, diseased buffaloes had upregulation of the expression of mRNAs of all tested proinflammatory markers compared with those of controls. This intensive inflammatory reaction was simultaneously associated with a compensatory anti-inflammatory reaction, during which the anti-inflammatory cytokines, i.e., IL-10, was upregulated. The mechanism for stimulation of APP production has been documented by the proinflammatory cytokines (Baumann and Gauldie 1994). The authors added that the inflammatory cytokines are the primary inducers of

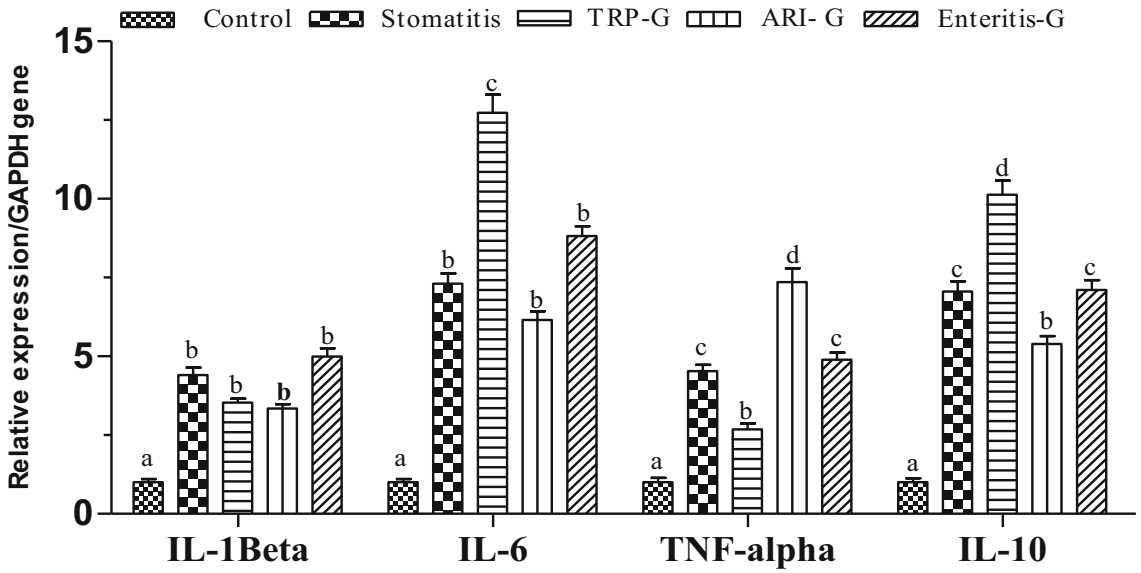

Fig. 1 Graphical presentation of real-time quantitative PCR analysis of the expression of IL-1β, *IL-6*, TNF-α, and IL-10 gene in buffaloes with some digestive disorders compared with clinically healthy controls. *Bars* labeled with different letters are statistically significant at $P < 0.05$

APP gene expression and each type initiates a different pattern of APP. Nearly similar findings concerning such elevated levels of TNF-α and IL-1β in inflammation and infection were previously observed in pigs (Reeth and Nauwynck 2000) or cattle (Pace et al. 1993; Horadagoda et al. 1994; Yoo et al. 1995; Morsey et al. 1999; Gruys et al. 2005). Similarly, the early and sustained expressions of acute phase cytokines have been reported to elevate in the airways and lung lesions of cattle experimentally infected with *Pasteurella haemolytica* where TNF-α, IL-1, and IL-8 mRNA were particularly expressed (Malazdrewich et al. 2001). A statistically significant high serum values of TNF-α, IL-1β, IL-6, and IL-10 have been recorded in adult buffaloes suffering from TRP (El-Ashker et al. 2013), being higher in non-survived cases than survivors (El-Ashker et al. 2014).

The results of the present study also demonstrated down-regulation of IgG and IgA gene expression in all diseased buffaloes compared with controls. However, these findings were away from those obtained in previous studies (Fiorentino et al. 1991; Hummelshoj et al. 2006). In those

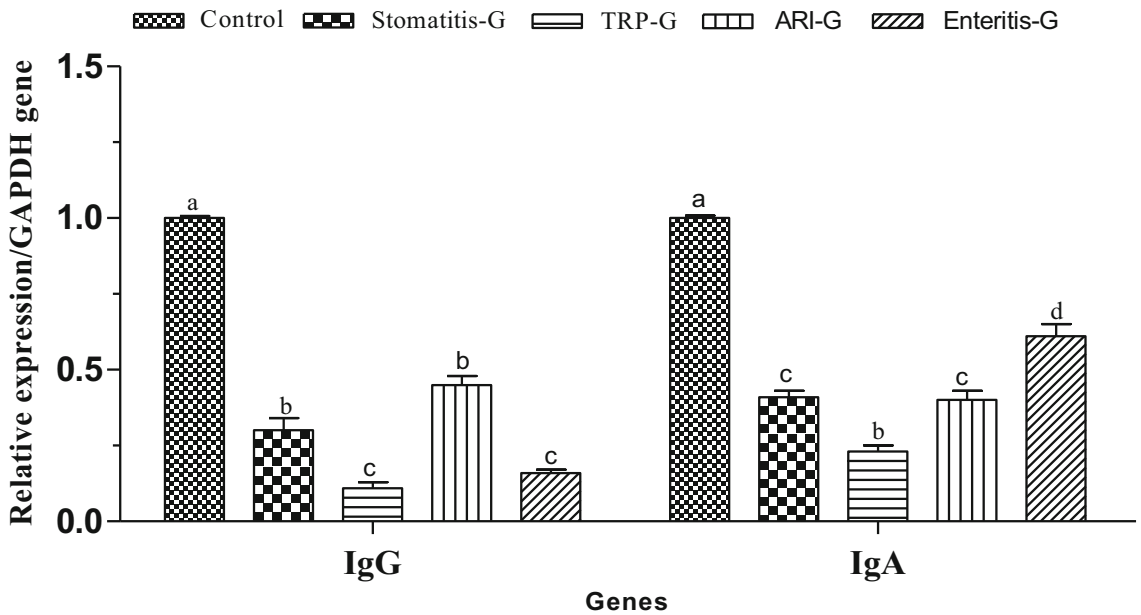

Fig. 2 Graphical presentation of real-time quantitative PCR analysis of the expression of IgG and IgA gene in buffaloes with some digestive disorders compared with clinically healthy controls. *Bars* labeled with different letters are statistically significant at $P < 0.05$

studies, the authors have suggested that IL-10 could positively affect the production of IgA and IgG and might support the regulation of B and T cell responses. The importance of low Ig and their relationship to the diseases of gastrointestinal origin has also been emphasized in dairy cattle with viral stomatitis (Cowan and Wagner 1972) or in calves with diarrhea caused by rotavirus, coronavirus, *Escherichia coli* F5 and *Eimeria* species (Balikci and Al 2014). Other reports have described the inheritance of maternal immunoglobulin G1 concentration by the bovine neonate (Muggli et al. 1984) or the effects of passive immunity on growth and survival in the dairy heifer (Robinson et al. 1988). But little is still known about the alterations of the gene expression of Ig in buffaloes with digestive disorders. The obtained findings were nearly similar to those reported recently in calves with diarrhea caused by rotavirus and corona virus and *E. coli* F5 (Balikci and Al 2014). The decreased levels of IgG and IgA indicate a state of immunosuppression. Some authors attributed the reductions of both variables to the binding of Ig to the microorganisms and their toxins to be neutralized thereby preventing the adherence of bacteria and virus to the target tissues (Andrews et al. 2004; Gershwin 2008). However, the downregulation of both genes being observed in the present study particularly animals with acute TRP needs further verification.

Conclusion

All diseased buffalo demonstrated distinctive inflammatory reaction with particular emphasis to those having acute TRP being associated with peak upregulation of IL-6 and IL-10 while IL-1β and TNF-α were mostly linked and upregulated with buffaloes having enteritis and acute rumen impaction, respectively. The diseased buffaloes were in a state of immunosuppression as indicated by downregulation of IgG and IgA gene expression with particular emphasis to buffaloes demonstrating acute TRP. The tested genes could have a vital role in the pathophysiologic mechanism of the underlying diseases. Further studies are needed to explore the ameliorative value of using supplementary agents to help mitigate the associated inflammatory reactions and help improve the immune status of buffaloes suffering from digestive disorders.

Authors' contributions MAY and MRE designed and coordinated the study. They were also responsible for data collection, analyses, and interpretation. MRE was responsible for writing and reviewing of the manuscript and corresponded with the journal. MFO responsible for clinical examinations, participated in samples collection, and took part in writing of the manuscript. All authors approved the final version of the manuscript for publication.

Compliance with ethical standards All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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