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ORIGINAL ARTICLE

Expression and Localization of Aquaporin-1 Along the Intestine of Colostrum Suckling Buffalo Calves

A. De Luca[1], G. Vassalotti[1], A. Pelagalli[2,3]*, M. E. Pero[1], C. Squillacioti[1], N. Mirabella[1], P. Lombardi[1] and L. Avallone[1]

Addresses of authors: [1] Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via Veterinaria, Naples 80137, Italy;
[2] Department of Advanced Biomedical Sciences, University of Naples Federico II, Via Pansini 5, Naples 80131, Italy;
[3] Institute of Biostructures and Bioimages, National Research Council, Via De Amicis 95, Naples 80131, Italy

***Correspondence:**
Tel.: +39 328 3515414;
fax: +39 081 7442092;
e-mail: alpelaga@unina.it

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Adriana De Luca, Alessandra Pelagalli and Caterina Squillacioti contributed equally to this work.

Summary

Aquaporin-1 (AQP1), a six-transmembrane domain protein, belongs to a highly conserved group of proteins called aquaporins known to regulate permeability across cell membranes. Although the role of AQP1 has been extensively studied, its specific activity along the gastrointestinal tract in animals during early postnatal development is poorly known. This study investigates the expression of AQP1 mRNA and protein in the small and large intestine of water buffalo calves after colostrum ingestion using reverse transcription–polymerase chain reaction (RT-PCR), Western blotting, and cellular localization of AQP1 by immunohistochemistry. Our results revealed AQP1 immunoreactivity and the presence of the corresponding mRNA in all the examined tracts of the intestine but with a different cellular localization. Western blotting confirmed the presence of AQP1, with a more intense band in colostrum-suckling animals. These findings offer insights into AQP1 expression in the small and large intestine, suggesting its involvement in osmoregulation in gastrointestinal physiology particularly during the first week after birth in relation to specific maturation of intestinal structures.

Introduction

Aquaporins (AQPs) are an extended family of relatively small intrinsic membrane proteins expressed in different mammalian tissues, which play a specific role in many physiological processes including water and small solute transport (Agre et al., 1998; Matsuzaki et al., 2002). To date, twelve different AQPs (0–11) have been identified and sequenced in mammals (Hatakeyama et al., 2001; Gorelick et al., 2006).

Functionally, these proteins have been classified into two subfamilies: aquaporins and aquaglyceroporins. Aquaporins are specialized in the transport of water and include AQPs 0, 1, 2, 4, 5, 6 and 10. In contrast, aquaglyceroporins transport not only water but also small molecules and include AQPs 3, 7, 8 and 9 (Verkman and Mitra, 2000). Newly discovered AQP11 shows low similarity to existing groups and may be a new superfamily (Gorelick et al., 2006).

In the past decade, many studies have focused on the expression and localization of different AQPs in numerous tissues of many mammalian species to define their specific function and thus assign a hypothetical role within each tissue (Ma and Verkman, 1999). Various studies using immunohistochemistry and other functional analyses have focused on AQP expression along the gastrointestinal tract (GT) given the activity of such tissues in regulating absorptive and secretive processes (Matsuzaki et al., 2004). Indeed, abundant fluid is transferred through the epithelial cell layer along the digestive system. Therefore, aquaporins appear to be involved in transepithelial water movement driven by osmotic gradients, although their specific functional role has not been unequivocally proven (Ma and Verkman, 1999).

Whilst several aquaporin families are known to exist, in mammalian species, the ubiquitous and extensively studied family is AQP1. It consists of six transmembrane domains connected by five loops and has its NH2 and COOH terminus located intracellularly (Murata et al., 2000). Although its localization along the gastrointestinal tract in humans and rats as in other vertebrates such as fish (Koyama et al., 1999; Cohly et al., 2008; Deane et al., 2011) appears to have a different pattern of distribution, the protein performs a general osmoregulatory role of water current.

However, the functional anatomy of the gastrointestinal tract has not been extensively studied in the context of water transport across endothelial and epithelial barriers and bulk fluid movement in lactating animals. Consequently, very little published information is available. This applies especially to ruminant species where the intestine performs several activities due to secretions of substances and absorption processes particularly related to tissue adaptive mechanisms occurring during the early postnatal development phase. In particular, after a meal, along the different tracts of the intestine, a large amount of water and other molecules are transported, thereby regulating the osmotic balance of intestinal contents.

The present study explores the expression and localization of AQP1 along the small and large intestine of neonatal buffalo calves after colostrum suckling. This animal species was chosen, as it is now widely bred in Italy. Buffalo farms play a central role in the regional economy in some areas of southern Italy for meat, milk production and fresh mozzarella cheese. The main factor that has contributed to the development of buffalo farming in the past few years has been the increase in mozzarella consumption both in Italy and overseas. Due to the favourable climatic conditions and to considerable economic interest, most of the increase has occurred in southern Italy (Borghese, 2013).

The choice of AQP1 was based on its extremely wide distribution in the intestine of different mammalian species. The expression of AQP1 mRNA and protein was studied by RT (reverse transcriptase) PCR and immunoblotting, and cellular localization was performed by immunohistochemistry.

There are two main advantages to be gained from this study. First, the results may lead to better development of management strategies to minimize calf mortality and enhance growth to maintain the animals in question in good health. Further, it could offer insights regarding nutritional regimens and feeding management systems for these animals reared for distinct purposes, namely for milk and meat production.

Materials and Methods

Animals and tissue sample collection

Fifteen healthy neonatal buffalo male calves from a single dairy farm in southern Italy were used in the study. Five calves were sacrificed at birth. The remaining ten animals were fed for 1 day with 3 l of mother colostrum using a needling bottle according to the farm's standard practice. On the second day, the animals were divided into two groups (five subjects each): the animals in first group continued to be fed with mother colostrum for 1 week; those in the second group were fed with buffalo milk in the same way. An additional group of five adult male buffaloes (15 months old) was used to evaluate potential age-dependent changes. The animals were killed in a public slaughterhouse. All procedures were conducted in accordance with EU Directive 2010/63 on the protection of animals used for scientific purposes.

The abdominal cavity was opened and the gastrointestinal tracts (GIT) were removed. Tissue samples of the small (duodenum, jejunum, ileum) and large intestine (caecum and colon) were immediately harvested and processed for fixing (see Immunohistochemistry) or snap frozen in liquid nitrogen and subsequently stored at −80°C until use for total RNA isolation and protein extracts. All procedures complied with Italian legislation regarding the use of animals in research.

Immunohistochemistry

Fresh segments of intestinal tissues were fixed by immersion in Bouin's fixative (6–24 h), processed for paraffin embedding in a vacuum and cut at a thickness of 5–7 μm. The avidin–biotin–peroxidase complex (ABC) method was performed using the Vectastain ABC kit (PK-4000 – Vector Laboratories, Burlingame, CA, USA). Paraffin sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. After the quenching of endogenous peroxidase activity in water containing 3% hydrogen peroxide for 30 min, nonspecific binding was blocked by treatment with 1.5% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 30 min.

Polyclonal goat anti-AQP1 (sc-9879, Santa Cruz Biotechnology, CA, USA) was applied to the sections at a dilution of 1:200 incubated in a moist chamber overnight at 4°C. After the sections had been washed three times in PBS, biotinylated anti-goat IgG (BA-5000,Vector Laboratories, Burlingame, CA, USA) was applied at a dilution of 1:200. The sections were again incubated for 30 min at room temperature (RT). Freshly prepared ABC reagent

(Vector Laboratories, Burlingame, CA, USA) was applied and incubated for 30 min after three washes in PBS. The localization of the immunoreactions was visualized by incubating the sections for 5 min in freshly prepared diaminobenzidine–nickel solution (Vector Laboratories). The specificity of the immunoreactions was tested by replacing the primary antibody with buffer. No immunoreaction was detected in control tests.

Five slides (one slide selected every ten according sequential thickness) for each intestinal tract (duodenum, jejunum, ileum, caecum and colon) from each animal were independently evaluated by two observers using a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

Western immunoblot analysis

Tissue samples were homogenized in 1× RIPA lysis buffer (0.1 mM PBS, 1% Nodinet P-40, 0.1% Sodium Dodecyl Sulphate 0.05% (SDS) deoxycholate, 1 μg/ml leupeptin and 1 μg/ml phenylmethylsulphonyl fluoride (PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16 000 × ***g*** for 20 min at 4°C. Aliquots of the supernatant were subjected to SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (12.5% polyacrylamide) under reducing conditions. Proteins were transferred (2 h at RT, 200 V) onto immunoblot nitrocellulose transfer membrane (PROTRAN, BioScience, Dassel, Germany) using a mini-transblot apparatus (Bio-Rad, Hercules, CA, USA). After 1 h blocking with blocking buffer (5% BSA and 0.3% Tween 20 in PBS), the membrane was incubated for 1 h at room temperature (RT) with anti-AQP1 (1:200) (Santa Cruz Biotechnology, CA, USA), and then washed three times and incubated for 1 h at RT with peroxidase-conjugated donkey anti-goat IgG (sc-2020 Santa Cruz Biotechnology, CA, USA, diluted 1:2000 in blocking solution). Marker proteins (coloured protein molecular weight markers; Prosieve, Lonza, Rockland, ME, USA) were used to estimate the molecular weight of each band. To monitor loading of gel lanes, the blots were stripped and re-probed using an anti β-actin monoclonal antibody (JLA20 CP01, Calbiochem, San Diego, CA, USA). Specific bands were visualized by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Thermo Scientific Pierce, Rockford, IL, USA), and the image was acquired with the Kodak Gel Logic 1500 imaging system (Celbio, Milan, Italy).

RNA extraction, cDNA synthesis, RT-PCR and sequencing

Samples of intestinal tracts were taken, immediately frozen by immersion in liquid nitrogen (−173°C) for 10 s, and individually homogenized in ice-cold TRI-Reagent (Sigma, St. Louis, MO, USA) using an Ultra-Turrax homogenizer. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in RNAase-free DEPC water. Total RNA was measured with an Eppendorf Biophotometer (Eppendorf AG, Basel, Switzerland). For cDNA synthesis, 1 μg of total RNA was retrotranscribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) and random hexamers as primers. For PCR reactions, specific primers for buffalo AQP1 were designed from the published gene sequences (*Bos Taurus* aquaporin-1 Genbank accession number NM_174702) using the Primer Express software (PE Applied Biosystems). The sense and antisense AQP1 primers used were 5′- GTGGCCCTGGGA CATCTG-3′ and 5′- CGTCGGCATCCAGGTCATAC-3′, which amplify a 250-bp fragment, the sense and antisense β-actin primers used were β-actin for 5′-CAG CTC CTC CCT GGA GAA GA-3′ and β-actin rev for 5′-CTG CTT GCT GAT CCA CAT CTG-3′, which amplify a 480-bp fragment.

The PCR cycle conditions were as follows: 94°C (30 s), 60°C (30 s), 72°C (1 min) for 35 cycles and 72°C (5 min). The PCR products of buffalo AQP1 and β-actin were purified using GFX PCR DNA and Gel Purification Kit (code 28-9034-70, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sequenced. PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light. A sample without cDNA template was used to verify that the master mix was free of contaminants.

Results

Immunohistochemistry

The immunohistochemistry results are summarized in Table 1. At birth, AQP1-immunoreactivity (IR) was found, with the exception of the duodenum, in the endothelium of the vessels of both the small and large intestine (Fig. 1a,d,g; Fig. 2a,d). The density of AQP1-IR was greater in the large intestine than in the small intestine. After 1 week of colostrum ingestion, AQP1-IR was distributed in the endothelium throughout the intestinal tracts (Fig. 1b,e,h; Fig. 2b,e). The density was greater in the jejunum than in the other tracts. In addition, AQP1-IR was found in the enteric neurons, enterocytes and lymphoid cells of both the small and large intestine (Fig. 1b,e,h; Fig. 2b,b',e,e'). After 1 week of milk suckling, AQP1-IR was localized in the endothelium of all the intestinal tracts and rarely in the enteric neurons of the large intestine (Fig. 1c,f,i; Fig. 2c,f). In the adults, AQP-IR was distributed in the endothelium, enterocytes,

Table 1. Distribution of AQP1 immunoreactivity in the buffalo small and large intestine

	Duodenum				Jejunum				Ileum			
	Birth	Colostrum	Milk	Adult	Birth	Colostrum	Milk	Adult	Birth	Colostrum	Milk	Adult
Endothelium	–	++	++	++	+	+++	++	+++	+	++	++	++
Enterocytes	–	–	–	++	–	+	–	+++	–	+	–	++
Enteric neurons	–	–	–	–	–	++	–	–/+	–	–/+	–	+
Lymphoid tissue	–	–	–	+	–	+	–	+	–	+	–	+

	Caecum				Colon			
	Birth	Colostrum	Milk	Adult	Birth	Colostrum	Milk	Adult
Endothelium	++	++	++	++	++	++	++	++
Enterocytes	–	–	–	+++	–	++	–	+++
Enteric neurons	–	+	–/+	+	–	+	–/+	+
Lymphoid tissue	–	+	–	+	–	+	–	+

–, undetectable; –/+, rare; +, low density; ++, medium density; +++, high density.

lymphoid tissue and enteric neurons of the different tracts of both the small (Fig. 3a–g) and large intestine (Fig. 3h–i).

Immunoblot analysis of the AQP1 protein

The expression of AQP1 protein in small and large intestine tracts of buffalo calves in different conditions (birth, colostrum, milk and adult) was investigated using a polyclonal AQP1 antibody raised against a peptide mapping at the C-terminus of AQP1. Analysis revealed a specific profile of the AQP1 protein (Fig. 4) with a band at 28 kDa corresponding to its molecular mass and other bands with higher molecular masses. In buffalo calves at birth, our experiments showed that AQP1 is present only as a faint band corresponding to a broad 45 kDa in the small and large intestine. By contrast, in animals which had received colostrum, in all examined tissues, AQP1 was expressed both as a band at 28 kDa and as multiple bands, respectively, of about 45 and 60 kDa probably corresponding to larger isoforms than those normally expected. A similar band profile was observed in animals which had received milk and in adult buffaloes with a different intensity for each band (Fig. 5).

RT-PCR analysis

RT-PCR analysis using AQP1 primer revealed the expression of AQP1 mRNA in the small and large intestine of buffalo calves, according to different animal conditions (birth, colostrum, milk), and of adult buffaloes (Fig. 6). The reaction revealed a single band of the expected size (250 bp) in all the examined intestinal tracts. To verify the efficiency of the reverse transcription (RT) and to exclude genomic DNA contamination, a fragment of β-actin cDNA (GenBank accession no. NC_007326) was amplified and sequenced with primers designed to span an intron β-actin for 5′-CAG CTC CTC CCT GGA GAA GA-3′ and β-actin rev for 5′-CTG CTT GCT GAT CCA CAT CTG-3′.

Discussion

In this work, we describe the expression and localization of AQP1 along the tracts of the small and large intestine of buffalo calves. The results obtained by immunohistochemical analysis supported also by Western blotting show the presence of the protein in different regions of the intestine, albeit with a particular expression in animals which had suckled colostrum. RT-PCR experiments revealed that AQP1 mRNA was expressed along the tracts of the small and large intestine of buffalo calves, confirming the immunohistochemical staining data. Prior to this study, no data were available regarding the expression of AQP1 mRNA in the buffalo intestine. Previous studies demonstrated the presence of AQP1 mRNA in the intestine of the pig (Jin et al., 2006) and rat (Koyama et al., 1999).

Tracts of the small and large intestine are known to participate differently in regulatory mechanisms of absorption and secretion, mobilizing a great movement of water flow and other molecules. The involvement of different transport pathways for water movement across the intestinal epithelium has acquired ever-growing importance in recent years since several AQPs have been identified in the digestive system of mammals (Matsuzaki et al., 2002). Our results suggest that AQP1 may be involved in different mechanisms of fluid regulation along

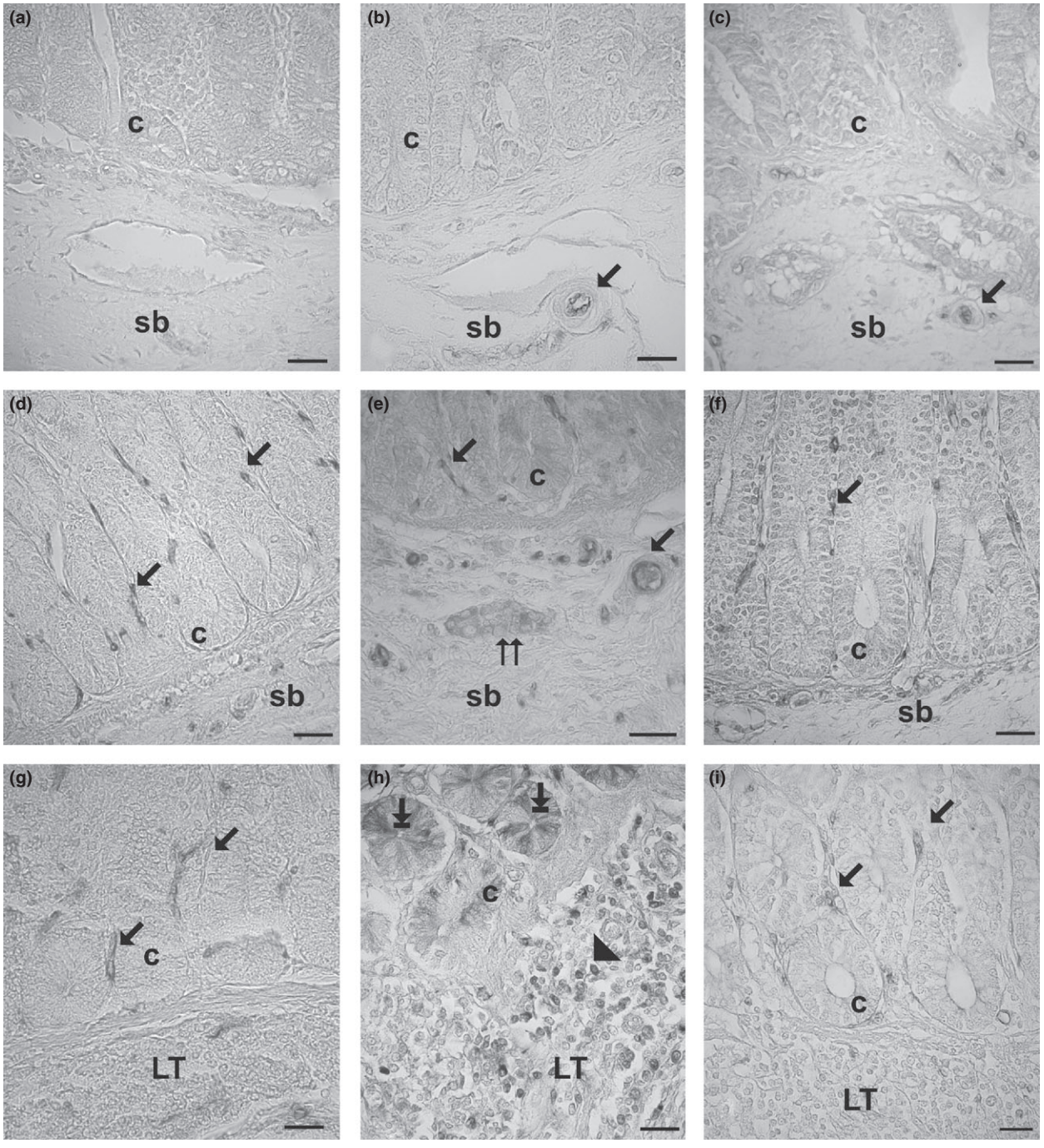

Fig. 1. Distribution of AQP1-immunoreactivity in the small intestine of the buffaloes at birth (a, d, g), and after 1 week of colostrum (b, e, h) and milk (c, f, i) suckling. (a–c) duodenum; (d–f) jejunum; (g–i) ileum; ↑ positive endothelium; positive lymphoid cells; positive enterocytes; positive enteric neurons; sb tela submucosa; c crypts; LT lymphoid tissue; scale bar 25 μm.

the intestine. In addition to AQP1, other AQPs may also be involved, but their identification lay beyond the scope of this study. Our results could be explained by the fact that colostrum can stimulate intestinal development (epithelial cell proliferation, enhanced villus size) (Roffler et al., 2003). In this case, it could, similarly to the development of intestinal tissues, enhance the development of pore-channel AQP1 or increase the AQP1 pore-channel number as suggested by Blatter et al. (2001). Also Baumrucker et al. (1994) described a more intense effect

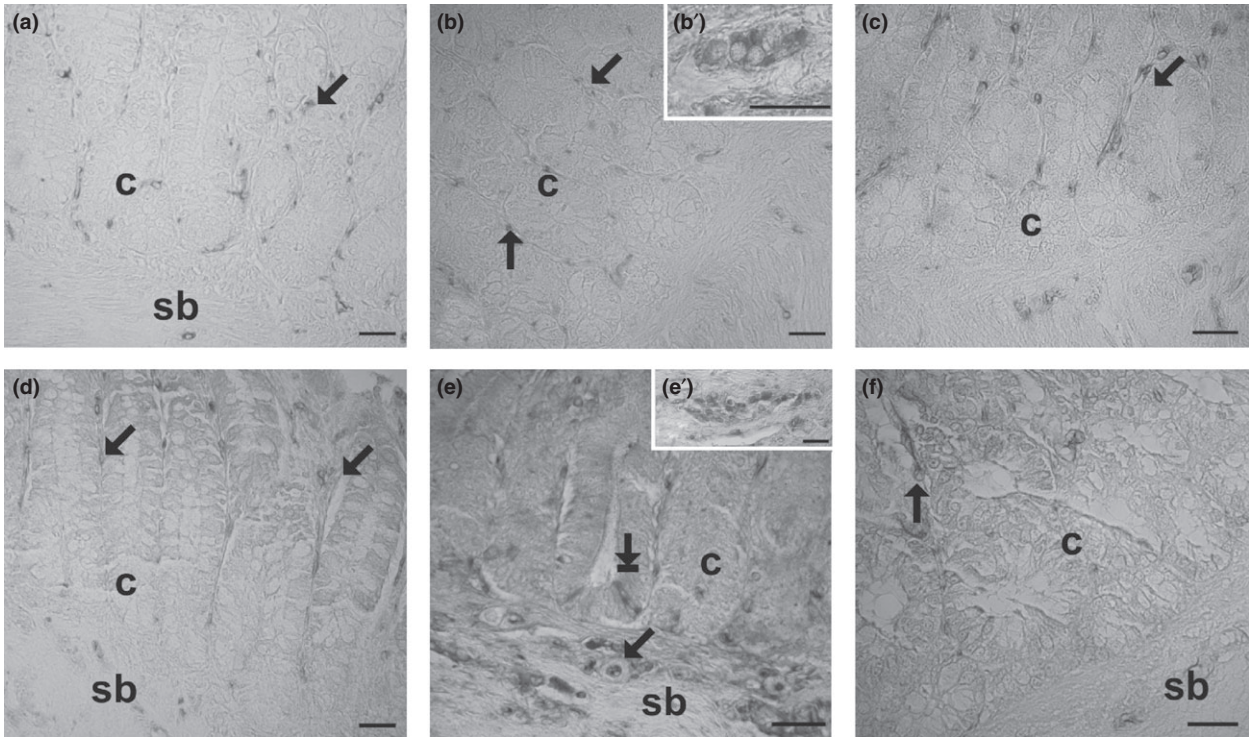

Fig. 2. Distribution of AQP1-immunoreactivity in the large intestine of the buffaloes at birth (a, d), and after 1 week of colostrum (b, e,) and milk (c, f,) suckling. (a–c) caecum; (d–f) colon (b′, e′) ganglia of the enteric nervous system; ↑ positive endothelium; positive enterocytes; positive enteric neurons; sb tela submucosa; c crypts; scale bar 25 μm.

of colostrum intake upon intestinal epithelia than mature milk. Others (Guilloteau et al., 2009) confirmed these theories on the basis of a different pattern of development in the intestinal tract in the first period after birth during which various enzymes such as cholecystokinin (CCK) can regulate intestinal cell activities (Biernat et al., 1999). Therefore, we cannot overlook the possibility that a simultaneous series of events and factors (nutrients, local hormones and immunomodulatory factors) could lead to a certain expression of aquaporins involved in regulating the passage of water and other molecules along the intestine. According to this hypothesis, AQP1 may only play a partial role in regulating water exchange, and the importance of other AQPs as well as other factors needs to be fully understood and investigated. Further, this hypothesis was recently described to analyse the role of several aquaporins in the mammary gland in the control of milk water content by regulating water homoeostasis (Mobasheri and Barrett-Jolley, 2014).

The results of immunohistochemistry reveal an equal distribution of AQP1 along the different intestinal tracts, albeit in distinct cellular locations, according to tissue maturation (animals at birth and adults). The particular distribution along the endothelial cells of capillaries is in good agreement with a previous report (Koyama et al., 1999) on rat specimens where the authors hypothesized a role of this protein for movement of water between interstitial fluid and lymphatic fluid in the digestive tract. Similar results reported by previous observations by Nielsen et al. (1993) described the presence of AQP1 in capillary endothelium rather than tissue parenchyma. The authors proposed that water intestinal absorption could occur by diffusional or paracellular mechanisms with rapid dilution into vascular spaces driven by increased oncotic pressures within the lumens of intestinal lacteals and capillaries. The presence of AQP1 along the crypts of the intestine is in agreement with previous studies on AQP4 expression in the rat gastrointestinal tract (Koyama et al., 1999). This localization could be related to fluid absorption activity (Naftalin and Pedley, 1999). According to the latter, solutes are transported actively out of the crypt lumen across a relatively water-impermeable crypt barrier to create a hypertonic interstitial space (Naftalin and Pedley, 1990; Naftalin, 1994). In addition, the presence of AQP1 in the lymphoid tissue of the gastrointestinal tract of the buffalo fed colostrum and of the adult animals is in accordance with a previous study on the human lymphocytes where the authors proposed that

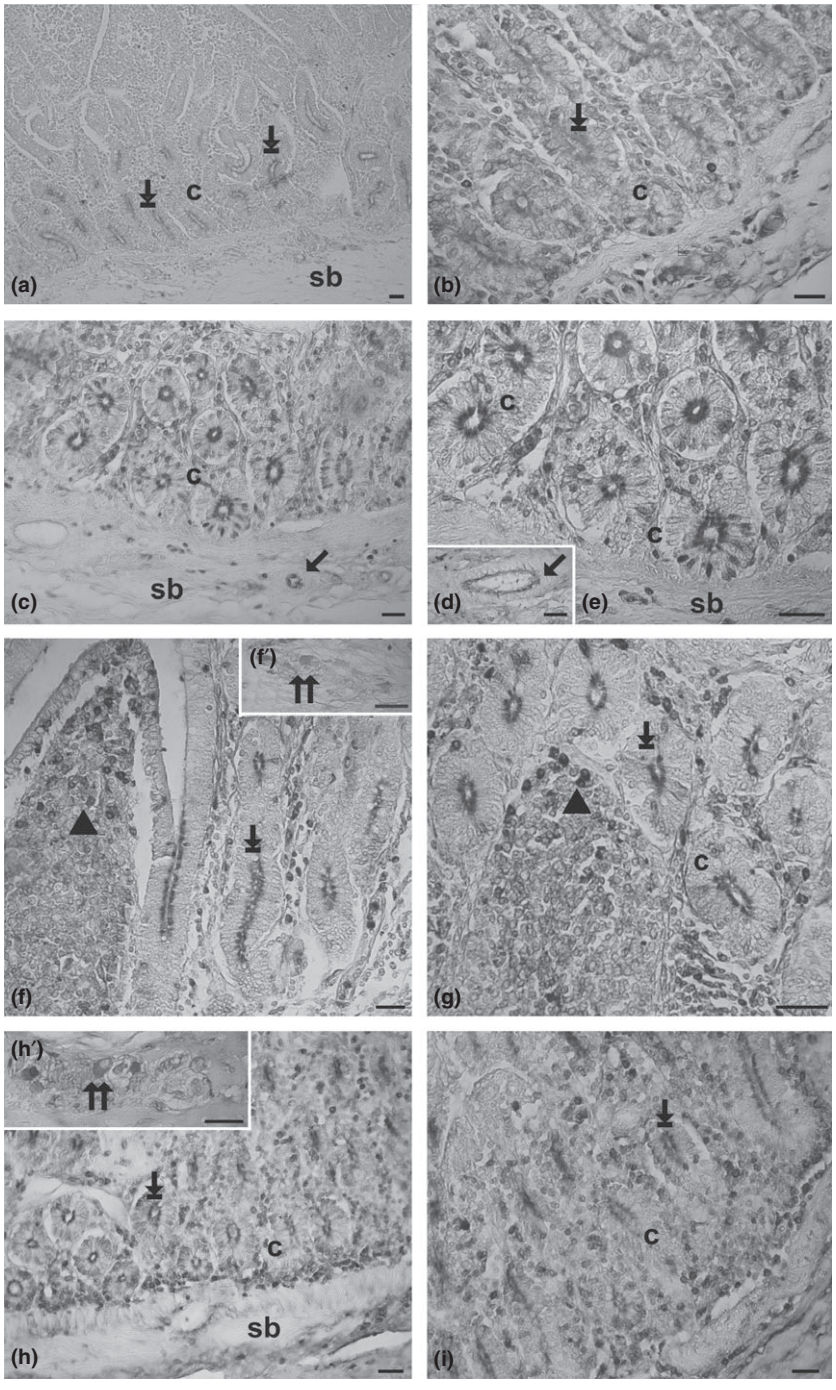

Fig. 3. Distribution of AQP1-immunoreactivity in the small (a–g) and large (h–i) intestine of the adult buffaloes. (a–b) duodenum; (c–e) jejunum (f–g) ileum; (h) caecum; (i) colon; (f′, h′) ganglia of the enteric nervous system ↑ positive endothelium; positive enterocytes; positive lymphoid cells; positive enteric neurons; sb tela submucosa; c crypts; scale bar 25 μm.

AQP expression patterns may be used as a marker to study lymphocyte activation and proliferation (Moon et al., 2004). Thus, our findings suggest a possible involvement of these lymphoid cells in intestinal immunity.

Another interesting result obtained, albeit not one of the aims of the present work, was the immunohistochemical presence of AQP1 in enteric neurons of the small and large intestine. This result is in agreement with recent studies conducted on rat (Nagahama et al., 2006) and sheep (Arciszewski et al., 2011). However, the exact localization and cellular distribution of the protein in the enteric system as well as its specific physiological role are still to be investigated in buffalo. However, the presence of AQP1 in enteric neurons seems well correlated with the role of such a system in regulating fluid homoeostasis

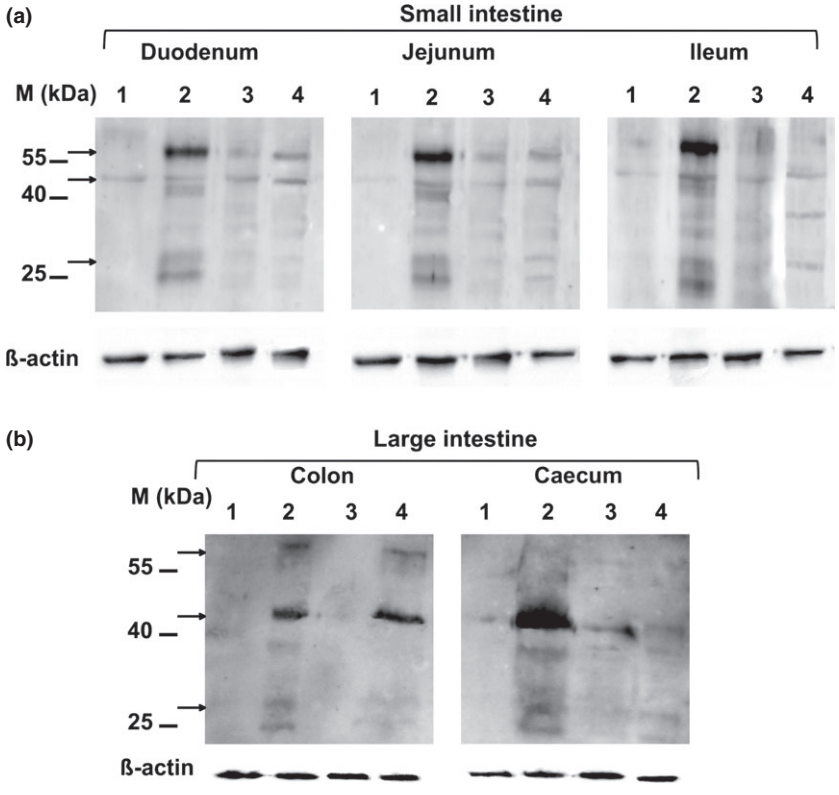

Fig. 4. Western blot analysis for AQP1 protein expression in different tracts (duodenum, jejunum and ileum) of small intestine (a) and large intestine (b) (caecum and colon) in animals in different conditions (birth, colostrum, milk and adult). Lane 1. Birth; Lane 2. colostrum; Lane 3. milk; lane 4. adult. β-actin was used as a loaded control.

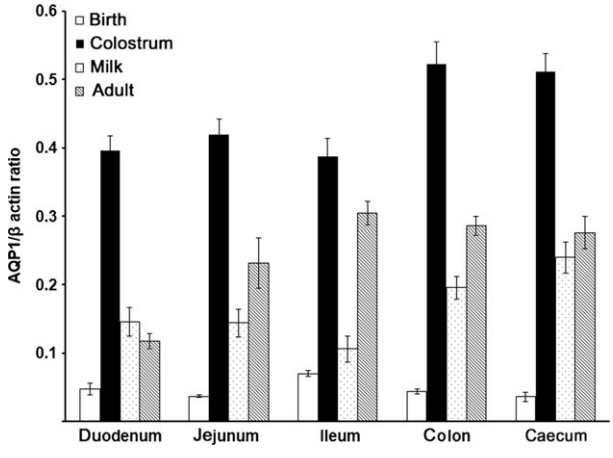

Fig. 5. Semi-quantitative analysis of AQP1 expression in the small and large intestine in animals in different conditions (birth, colostrum, milk and adult). Averaged data from five animals from each group on three different blots for each intestine tract are expressed as ratios with the corresponding value for β-actin. Data are presented as means ± standard error (SE).

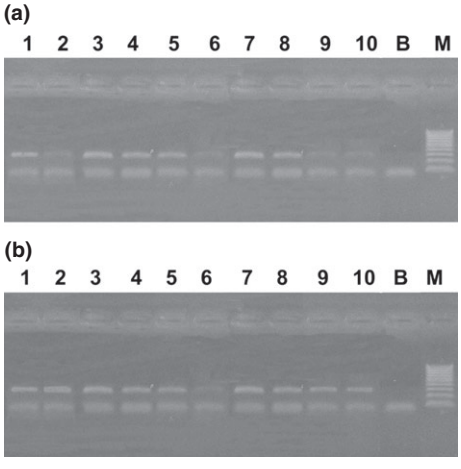

Fig. 6. AQP1 expression by RT-PCR (a–b). AQP1 (250 bp fragments) was expressed in duodenum, jejunum, ileum, caecum and colon of animals at birth [Panel (a), lane 1–5], animals after 1 week of colostrum suckling [panel (a) lane 6–10], animals after 1 week of milk suckling [panel (b) lane 1–5] and adults [panel (b) lane 6–10] (b) Control; M: molecular markers of 100-bp ladder.

by the transport of different molecules across the intestinal epithelium by monitoring osmotic pressure.

In our results obtained by Western blot analysis, the simultaneous presence of different bands (28–55 kDa) of AQP1 confirmed data evidenced by previous studies on the avian intestine (Casotti et al., 2007). The authors suggest the expression of a glycosylated form of AQP1 as products of post-translational processing (Laforenza et al., 2005).

In conclusion, this study provides the first report of the presence of AQP1 along the buffalo calf intestine. The data showing a different localization of AQP1 along the cells of examined intestinal tracts are consistent with the role performed by this channel protein in transporting water and small solutes, thereby contributing to the regulation of fluid homoeostasis and pressure osmoregulation by enteric neuronal control. Further functional studies are required to establish the specific role of this protein along the intestinal tract to ascertain the specific adaptation mechanisms occurring along the tissues during the early postnatal period.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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