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Constitutive expression pattern of ITGB6 receptor gene in the air pathways of buffalo

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ABSTRACT

Integrin beta 6 (ITGB6) is one of the important integrin family proteins, required for viral entry in their respective host. The constitutive expression of ITGB6 was reported in cattle and sheep but not in buffalo. The present study is first of its kind to characterize the constitutive expression pattern of ITGB6 in the air pathways of buffalo (*Bubalus bubalis*). In the present study, we characterized the constitutive expression pattern of ITGB6 in the air pathways of buffalo. Our data demonstrated that mRNA transcript and concentration of ITGB6 are significantly higher in tongue tissues compared to trachea, bronchi and lung. The study may highlight that oral mucosa of buffalo are enriched with integrin receptor genes, which are the target of certain viral entry site.

Key words: Buffalo, Expression, ITGB6

Integrins are heterodimeric adhesion receptors that contribute in a variety of cell–cell and cell–extracellular matrix protein interactions. Integrin beta 6 is a subunit of avb6 belonging to a subfamily of integrins, which recognize a common arginine–glycine–aspartate (RGD) motif present in VP1 gene of FMD virus (Wang *et al.* 2015). Monaghan *et al.* (2005) reported that integrin-avb6 expression is predominant in the stratified squamous epithelium of tongue, ventral soft palate, interdigital skin and coronary band of cattle, where FMDV lesions are most commonly observed. Similarly, Brown *et al.* (2006) reported that the expression of integrin-avb6 is restricted to epithelial cells in the sheep, and that it is expressed at sites of known FMDV replication including the basal layers of the stratified squamous epithelium of the oral mucosa and pedal coronary band (PB). In the present study, we analyzed the constitutive expression profile of ITGB6 gene in buffalo (*Bubalus bubalis*) air pathway (tongue, trachea, bronchi and lung).

MATERIALS AND METHODS

Collection of tissue samples: Tissue samples (tongue, trachea, bronchi and lung) were collected from buffalo slaughterhouse immediately after slaughter. Around 500 mg of each tissue sample (6 numbers of each tissue) were placed in 10 ml of RNA later for RNA extraction and remaining stored in phosphate buffer saline for extraction of protein. Samples were stored in –80°C until use.

Determination of ITGB6 cDNA concentration in different tissue samples: Tissues were ground thoroughly with tissue homogenizer. Total RNA was isolated from each ground tissue samples using Cold trizol method, following manufacturer recommendations. The integrity of the RNA was checked by visualization of 18s and 28s ribosomal bands on an agarose gel. Reverse transcription of total RNA was carried out using a cDNA synthesis kit as per the manufacturer's recommendations employing the M-MuLV reverse transcriptase and random primers. Details of the primers used for this study are given in Table 1. The cDNA product was stored at –20°C. A diluted 1:10 solution of the

Table 1. Sequences of primer sets used for the present experiment

Gene	Primer sequence
ITGB6	Forward:5' GAAGAATGTGTTGACA AATGCAAAC3'
	Reverse: 5' CAGAGAACAGGAAAC AAAGCTATCC3'
Beta actin	Forward: 5' AGTTCGCCATGGATGATGA3'
	Reverse: 5' TGCCGGAGCCGTTGT3'

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cDNA was used to perform the downstream PCR amplification using the ITGB6 specific primers. The concentrations of the cDNA were evaluated by adding 1 µl SYBER GREEN-I dye to each amplified product from different tissue samples. Changing of colorimetric reaction observed under UV light.

Analysis of ITGB6 mRNA transcripts in different tissue samples: Real-time qPCR was used to elucidate the differential expression of ITGB6 gene among different groups. Real-time fluorescence detection method (PCR) was used to quantify the RNA expression of the ITGB6 receptor gene. Beta actin was used as an endogenous control. Amplification was performed for 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Samples were quantified by the ΔΔCt method (Livak and Schmittgen 2001). The expression values obtained were normalized against Beta actin, allowing the comparison of samples independently of the amount of total input cDNA.

Detection of ITGB6 concentration in different tissue samples: Frozen tissue samples were rapidly thawed at 37°C, and further homogenized in tissue lysis buffer with tissue homogenizer. Concentrations of ITGB6 in different tissue samples were determined by an indirect ELISA assay using standard. The total protein levels of homogenized tissue extracts were measured with a BSA protein assay kit. The cells were first washed with PBS and lysed with lysis buffer. At first, 96-well plastic microtitre ELISA plates were coated for overnight with coating buffer (pH 9.6) containing antigen. The plates were washed with PBS containing 0.05% Tween-20 (PBST) for 3 times. Blocking was done with 2% bovine serum albumin for 1 h at 37°C. Again washing was done with PBST for 3 times. A 1 h incubation was given at 37°C with primary antibody i.e. monoclonal anti-ITGB6 antibodies produced in mouse. After washing with PBST, the plates were incubated for 1 h at 37°C with conjugated secondary antibody i.e. peroxidase conjugated antimouse Fab specific IgG raised in goat. Substrate o-phenylenediamine dihydrochloride diluted in H_2O_2 were added and after development of colour, the optical density of the samples was measured at 450 nm within 30 min using a microtitre plate reader.

I*mmune dot blotting to detect extent of ITGB6 agglutination in different tissues:* The extent of agglutination reaction of the ITGB6 antigen in different tissue samples was determined by the standard protocol of immune dot blotting described elsewhere. Briefly, 2–3 µl of concentrated antigen of different tissue samples were placed on NCM papers, air dried, blocked with 2% BSA and washed three times with PBS. Further, incubated with primary antibody, i.e. monoclonal anti-ITGB6 antibodies (1:10,000) produced in mouse for 1 h. After washing with PBS, incubated for 1 h at 37°C with peroxidase conjugated anti-mouse secondary antibody (1:2000). Detection was performed using metal enhanced DAB substrate kit as per manufacturer's protocol.

I*mmune fluorescent assay to detect ITGB6 in different tissue samples:* Drop of lysed tissue sample extracts were smeared in clean grease free slides. Cells were fixed by incubating with freshly prepared 3.7% (v/v) formaldehyde in PBS for 20 min. Cells were treated with permeabilization buffer (0.5% Triton X-100, 0.2 µg/ml EDTA in 1 × PBS) for 10 min followed by 3 rinses with PBS. Non-specific sites were blocked with blocking reagent (2% BSA) at 4°C. Cells were then incubated with monoclonal anti-ITGB6 antibodies produced in mouse for 2 h at 37°C, washed 3 times with PBS, and incubated with FITC conjugated rabbit anti-mouse IgG antibody. The cover slips were washed, mounted on slides with anti-fade DPX. Fluorescent micrographs of cells were taken at 20× magnification in a microscope with a fluorescence illuminator system. Cells from at least 5 microscopic fields were counted.

Statistical analysis: All determinations were performed in triplicate. Data are presented as mean ± SEM and analyzed by using SPSS statistical program. Significant differences were determined by one-way analysis of variance (ANOVA) using the SPSS program.

RESULTS AND DISCUSSION

The beta 6 integrin (ITGB6) was identified as a component of fibronectin receptor in cultured epithelial cells. We attempted to detect the expression profile of ITGB6 in different respiratory tissues of buffalo at transcript and protein level.

Integrin beta 6 is one of the significant biologically active proteins responsible for foot and mouth disease (FMD) virus host interaction. Virus attachment to the integrin receptors is facilitated by an arginine–glycine–aspartic acid (RGD) tripeptide located on VP1 loop of FMDV (Singh *et al.* 2014). Constitutive expression of integrin avb6 in the airway epithelia with apparent absence of inflammation was earlier reported in cattle (Monaghan *et al.* 2005) and sheep (Brown *et al.* 2006).

cDNA concentration was determined by adding SYBER GREEN DYE I in the amplified product of buffalo ITGB6 gene from different tissue samples (Fig. 1). Results demonstrated that the colour intensity was comparatively higher in buffalo tongue tissue compared to trachea, bronchi and lung. Spectrophometer reading of the amplified products showing a higher concentration of cDNA copy number in tongue tissue followed by trachea, bronchi and lung (Fig. 1).

Real time PCR based quantification of ITGB6 from different tissues showed that mRNA transcripts were significantly ($P<0.05$) higher in tongue tissue (4.5±0.06) compared to trachea (3.2±0.04), bronchi (2.8±0.05) and lung (2.3±0.05). Brown *et al.* (2006) reported that integrin-b6 transcripts were detected throughout the upper gastrointestinal tract, including the oral mucosa where, once infection is established, productive FMDV lesions are usually found (Grubman and Baxt 2004). Similarly, studies reported that ITGB6 expression is comparatively higher in bovine tongue epithelial cells (Monaghan *et al.* 2005).

To identify the concentration of ITGB6 gene in different tissues of buffalo, we demonstrated the antigen-antibody

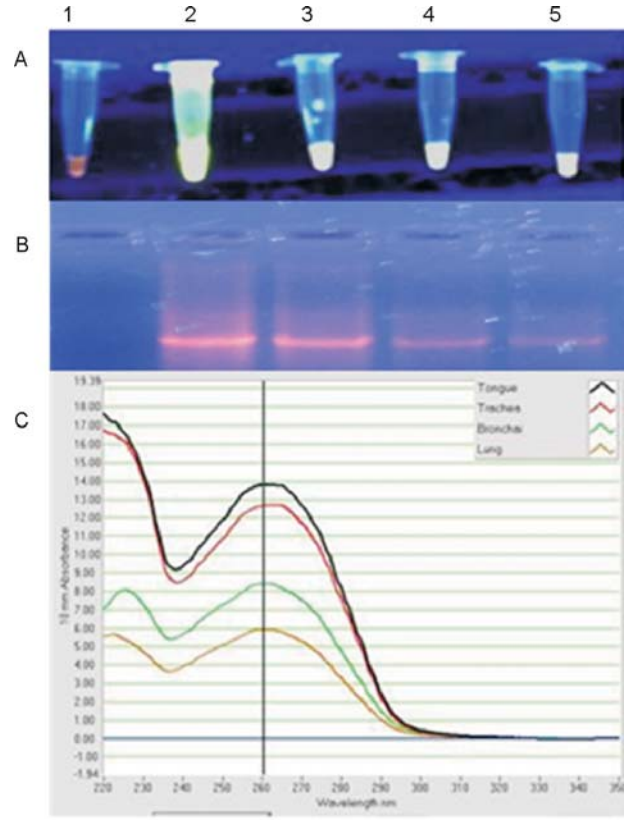

Fig. 1. Determination of cDNA concentration of ITGB6 gene in different tissue samples. **A.** SYBER Green I dye based visible detection of cDNA amplified product of ITGB6 gene in different tissue samples [1, NTC (No template control); 2, Tongue; 3, Trachea; 4, Bronchi; 5, Lung]. **B.** Amplified product of ITGB6 cDNA [1, NTC (No template control); 2, Tongue; 3, Trachea; 4, Bronchi; 5, Lung]. **C.** Spectrophotometer reading of amplified product of ITGB6 with different concentration.

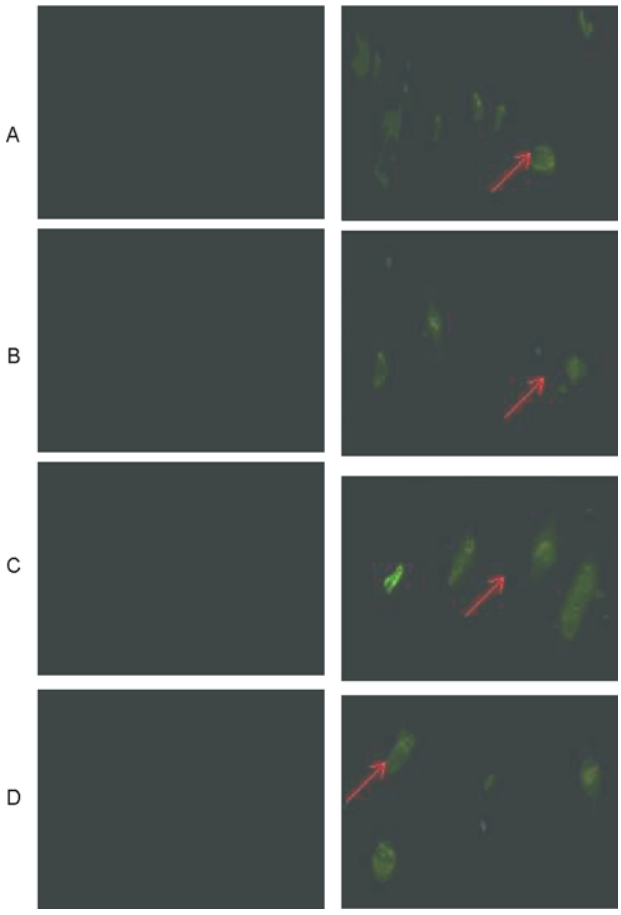

Fig. 3. Representative fluorescent micrographs of cells showing expression of ITGB6 gene in different tissue samples. **A.** Tongue; **B.** Trachea, **C.** Bronchi; **D.** Lung. Left panel is control.

interaction with mouse specific ITGB6 monoclonal antibodies. Fig. 2 revealed the antigen-antibody interaction by means of immune dot blot, where the agglutination was higher in tongue tissue compared to trachea, bronchi and lung. Concentration of ITGB6, detected by indirect ELISA, revealed that tongue (0.26±0.03) had significantly ($P<0.05$) higher concentration than trachea (0.19±0.01), bronchi (0.18±0.01) and lung (0.18±0.03). These studies indicated that integrinb6 expression is similarly distributed as described in transcript level.

Further, immune localization of ITGB6 gene in different tissue samples were performed by Immune fluroscent assay (Fig. 3). The study revealed that number of cells reacted with FITC conjugated secondary antibody was comparatively superior in tongue tissue than trachea, bronchi and lung.

Fig. 2. Immune dot blot assay for detecting ITGB6 concentration among tongue (1), trachea (2), bronchi (3) and lung (4) tissue of buffalo.

The preliminary study, which is first of its kind in buffalo indicated higher expression of ITGB6 in tongue tissue compared to trachea, bronchi and lung. This may conclude that, oral mucosa may be one of the important portal entry sites of the virus in buffalo. However, the exact portal site of the virus in buffalo yet to be described with i*n-situ* confocal studies.

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