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Expression of β-defensins pBD-1 and pBD-2 along the small intestinal tract of the pig: Lack of upregulation in vivo upon *Salmonella typhimurium* infection

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Abstract

Defensins are antimicrobial peptides that play an important role in the innate immune response in the intestine. Up to date, only one β-defensin (pBD-1), has been described in pig, which was found to be expressed at low levels in the intestine. We set-up a quantitative PCR method to detect the gene expression of pBD-1 and a newly discovered porcine β-defensin, pBD-2. Expression of pBD-1 mRNA increased from the proximal to the distal part of the intestine whereas pBD-2 expression decreased. The main gene expression sites for pBD-2 were kidney and liver, whereas pBD-1 was mainly expressed in tongue. The porcine small intestinal segment perfusion (SISP) technique was used to investigate effects of *Salmonella typhimurium* DT104 on intestinal morphology and pBD-1 and pBD-2 mRNA levels in vivo. The early responses were studied 2, 4 and 8 h post-infection in four separate jejunal and ileal segments. Immunohistochemistry showed invasion of the mucosa by *Salmonella* and changes in intestinal morphology. However, no concomitant changes in expression of either pBD-1 or pBD-2 were observed. We conclude that at least two defensins are differentially expressed in the intestine of pigs, and that expression of both defensins is not altered by *S. typhimurium* under these conditions.

Keywords: Porcine β-defensins 1 and 2; Small intestinal segment perfusion; Innate immunity; Defensin; *Salmonella typhimurium*

1. Introduction

Defensins comprise a major subclass of the family of antimicrobial peptides (AMPs). Hundreds of AMPs have been described in vertebrates, invertebrates, plants (http://www.bbcm.units.it/~tossi/pag1.htm) and recently even in a fungus (Mygind et al., 2005), the number of newly discovered AMPs is still growing fast. Although originally thought of as true antimicrobial peptides, capable of killing bacteria by membrane disruption, several additional roles in innate immunity have recently been assigned to AMPs. These include chemotactic activities for immature dendritic cells, memory T-cells and monocytes (Territo et al., 1989; Yang et al., 2000), stimulation of angiogenesis (Conejo-Garcia et al., 2004; Li et al., 2000) and mast cell activation to induce chemotaxis, histamine release and prostaglandin (PGD_2) production (Niyonsaba et al., 2001, 2002). Thus, besides being effector molecules of innate immunity, β-defensins also provide a link between innate and adaptive immunity.

The intestine is best seen as a complex and dynamic system where intestinal microbes symbiotically interact with host cells (Xu and Gordon, 2003). Antimicrobial peptides play an important role in microbe–host crosstalk, thereby maintaining intestinal microbiotic homeostasis. Increasing evidence suggests that decreased levels or malfunctioning of defensins in the intestine might lead to disturbance of this homeostasis resulting in

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intestinal diseases such as Crohn's disease and ulcerative colitis in human (Wehkamp et al., 2005a,b). Furthermore, an upregulation of intestinal defensins is observed as an early response to bacterial infections of the intestine (Zilbauer et al., 2005).

In pig, only one β-defensin, porcine defensin 1 (pBD-1) has been described up to date (Zhang et al., 1999). This defensin was found primarily in tongue mucosa but low mRNA levels were also found in other epithelial tissues including the intestine. Based on sequence homology of the cysteine residues, three other β-defensin mRNA sequences have been discovered in porcine Expressed Sequence Tags databases and have been assigned pBD-2–4 (GenBank™: AY506573, AY4605575 and AY4605576). Of these β-defensins, no data on structure, expression pattern or biological activity has been described to date.

pBD-1 is thought of as a constitutively expressed AMP, contrary to other inducible β-defensins, such as human β-defensins 2 and 3 (Harder et al., 2001; Liu et al., 1998), tracheal antimicrobial peptide (TAP) (Diamond et al., 1996) or bovine epithelial beta defensin (EBD) (Tarver et al., 1998). This is mainly based on in vitro experiments where no induction of pBD-1 could be observed in tongue epithelial cells upon infection with *Salmonella typhimurium* (Zhang et al., 1999). However, in other tissues, where lower levels of pBD-1 are observed, pBD-1 levels may be regulated by pathogenic bacteria. Especially in the intestine, a constitutive high expression of defensin (or other AMP) molecules could be undesirable because of the impact on the beneficial commensal bacteria in the intestinal microflora (Finlay and Hancock, 2004).

Salmonella enterica serovar *typhimurium* (hereafter *S. typhimurium*) is an important intestinal pathogen that causes several diseases in animals and humans. In pigs, it is mainly associated to enterocolitis, a disease very similar to gastroenteritis in humans (Darwin and Miller, 1999). *S. typhimurium* phagetype DT104, that is isolated with increasing frequency in clinical isolates, causes an additional public health threat because of its antibiotic resistance (Akkina et al., 1999). The effect of *S. typhimurium* on the regulation of antimicrobial peptides is slowly emerging. Recently, an eight-fold increase in pBD-1 mRNA expression was observed upon *S. typhimurium* infection in the porcine intestinal cell line IPEC J2 (Sang et al., 2005), also showing for the first time that upregulation of pBD-1 is indeed possible. In addition, *S. typhimurium* (or structural proteins of *S. typhimurium*) have been shown to upregulate expression of human β-defensins in vitro (Ogushi et al., 2001) and in vivo (O'Neil et al., 1999). These findings, and the fact that pBD-1 has been shown to possess antimicrobial activity against *S. typhimurium* in vitro (Shi et al., 1999), indicate a potential role for defensin upregulation in the intestine upon *S. typhimurium* infection as part of the total innate immune response.

In the present study, we describe the gene expression within the intestine and the tissue distribution for pBD-1 and the newly discovered β-defensin, pBD-2, mRNA. In addition, the effect of *S. typhimurium* DT104 infection on intestinal morphology and the early transcriptional pBD-1 and pBD-2 response (0–8 h) of pig intestinal mucosa is investigated using the small intestinal segment perfusion (SISP) technique.

2. Materials and methods

2.1. Materials

Double dye probes, primers and the one-step qPCR kit were purchased from Eurogentec, Belgium. All chemicals were of analytical grade.

2.2. Methods

Total cellular RNA was isolated from tissue by disruption of 100 μg tissue in 1.2 ml TRIzol® (Invitrogen Life Technologies) using Magnalyzer Beads (Roche Diagnostics GmbH, Germany). RNA isolation was performed as recommended by the supplier with an additional wash step with ice-cold 75% ethanol to remove the final traces of organic solvents. Purity and quality of the RNA extracts was checked on 1% agarose gels and using UV absorption at 260 nm/280 nm.

2.3. Animals

Crossbred (Yorkshire × Dutch Landrace) piglets between 28 and 35 days of age were used for the experiments. Prior to onset of the study, all pigs were determined to be free of culturable *Salmonellae* organisms in fecal samples, and no obvious clinical signs of enteric disease were observed throughout the course of the study. The animal experiment was approved by the local Animal Ethics Commission in accordance with the Dutch Law on Animal Experimentation.

2.4. Bacterial strain

The *S. typhimurium* strain used was isolated from a diseased pig, calf and child on a Dutch farm (Hendriksen et al., 2004). All three isolates were indistinguishable by phenotypic and genotypic methods, and were typed as *S. typhimurium* DT104 (a kind gift by Dr. Engeline van Duijkeren, Veterinary Microbiologic Diagnostic Centre, Utrecht University, The Netherlands).

2.5. Gene expression pattern of pBD-1 and pBD-2 in the intestine

From the intestine of five healthy 30-day-old piglets, eleven samples were taken along the duodenum (two samples), jejunum (six samples) and ileum (three samples). The distance between segments was 30 cm in the duodenum, 1.3 m in the jejunum and 60 cm in the ileum.

2.6. Small intestinal segment perfusion

The SISP was performed essentially as described by Nabuurs et al. (1993). Briefly, four pigs were sedated with 0.1 ml azaperone (Stressnil)/kg bodyweight; after 15 min, inhalation anaesthesia was performed with a gas mixture of 39% oxygen, 58% nitrous oxide and an initial 3% isoflurane; after 10 min, 2%

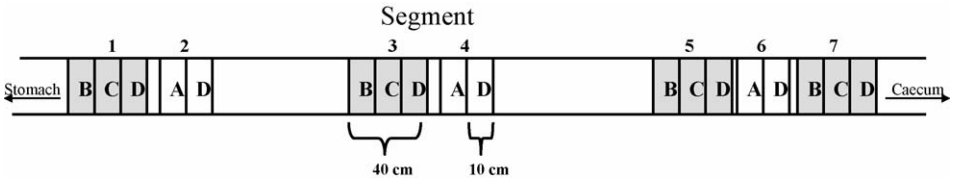

Fig. 1. Schematic presentation of the intestinal segments used in the SISP experiment. Segments were collected after: (A) 0 h, (B) 2 h, (C) 4 h and (D) 8 h. Shaded (odd numbered) segments were infected with *S. typhimurium* DT104. White (even numbered) segment were used as controls.

isoflurane. The abdominal cavity was opened and seven segments of 20 cm (control segments) or 40 cm (infected segments) were prepared by inserting a small inlet tube in the cranial site and a wide outlet tube into the caudal site of a segment. The first two segments were located in the proximal jejunum directly after the ligament of Treitz. Segments three and four were located in the mid-jejunum and segments five, six and seven covered most of the ileum. The location of the segments is schematically shown in Fig. 1. Segments 1, 3, 5 and 7 (initially 40 cm) were perfused for 1 h with peptone solution containing 10^9 CFU/ml of *S. typhimurium*, followed by perfusion with peptone only. Control segments (#2, 4 and 6) (initially 20 cm) were perfused with peptone only. Mucosal samples for histology and RNA isolation (10 cm) were taken at 0, 2, 4 and 8 h as indicated in Fig. 1, after which the tubing was reconnected, and perfusion was resumed. After sample collection at 8 h, the pigs were euthanised by barbiturate overdose.

2.7. Morphological characterization

Paraffin tissue sections were stained using Haematoxylin and Eosin according to standard procedures (Drury and Wallington, 1980) and each segment was visually scored for the following characteristics: presence of granulocytes in the mesentery, lymphatic vessels, lumen or serosa, swelling of submucosa or villi and the presence of abnormal villi. Scoring was done on a scale of 0–5, where 0 indicated no detection and a score of 5 indicated a high abundance of the specified characteristic throughout the whole section.

2.8. Immunohistochemistry

Invasion of *S. typhimurium* was established by immunohistochemistry on deparaffinised tissue sections, using a specific anti-O *S. enterica* serovar *typhimurium* antibody (1:200 in PBS + 2.5% BSA, 1 h at room temperature) essentially as described before (Tersteeg et al., 2002). Briefly, endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol. Normal goat serum (10%, v/v, Jackson Immunoresearch West Grove, PA, USA) + 2.5% BSA, was used to reduce non-specific background staining. After incubation with the secondary biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA, USA, 1:250 in PBS + 2.5% BSA) sections were treated with ABC/PO reagent (Vector) for 30 min. Between steps, sections were rinsed in PBS/Tween. Finally, the sections were incubated with DAB (3,3′-diamino-benzidine, Sigma), 0.05% in 50 mM Tris, pH 7.6, 0.03% hydrogen peroxide) for 10 min to visualize the antigen.

2.9. Quantitative PCR

A quantitative PCR reaction was set-up for three genes: porcine β-defensins 1 and 2 and hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes using the primers and probes depicted in Table 1. Probes were labelled with 5′ FAM (pBD-1 and HPRT) or 5′ Yakima Yellow (pBD-2) and 3′ Deep Dark Quencher 1 (all probes). All qPCR reactions were performed using one-step qPCR technology (Eurogentec), utilizing 0.1 ng RNA per reaction in 25 μl on a ABI Prism 7000 sequence detection system (Applied Biosystems, Weiterstadt, Germany), using the following cycling protocol: 30 min at 48 °C (RT step) and 10 min at 95 °C (denaturation); 42 cycles: 15 s at 95 °C and 60 s at 60 °C. Relative gene expression to the housekeeping gene HPRT was performed in order to correct for the variance in amounts of RNA input in the reactions. Additionally, the relative expression index (REI: $2_T^{-\Delta\Delta Ct}$) was determined for the SISP samples by comparison of the expression levels of the infected to the average expression levels of the control samples, with the index defined as 1.

2.10. Statistical analysis

Statistical analysis was performed using SPSS Version 10.0.5 for Windows. The defensin expression pattern in the intestine was analysed by the post hoc Tukey HSD test, while differences between infected and non-infected intestinal segments in the SISP experiment were investigated by the Student's paired *t*-test. Significant differences were defined as $p < 0.05$.

Table 1
Primers and probe sequences used for qPCR

		Sequence 5′ → 3′	Concentration (nM)
HPRT	Fw	tggaaagaatgtcttgattgttgaag	100
	Rev	atctttggattatgctgcttgacc	200
	Prb	acactggcaaaacaatgcaaacctgct	100
pBD-1	Fw	tgccacaggtgccgatct	100
	Rev	ctgttagctgcttaaggaataaaggc	100
	Prb	tttggagcacacttgcccggcata	800
pBD-2	Fw	ccagaggtccgaccactaca	300
	Rev	ggtcccttcaatcctgttgaa	200
	Prb	ctgcaacttctccccctgcccg	300

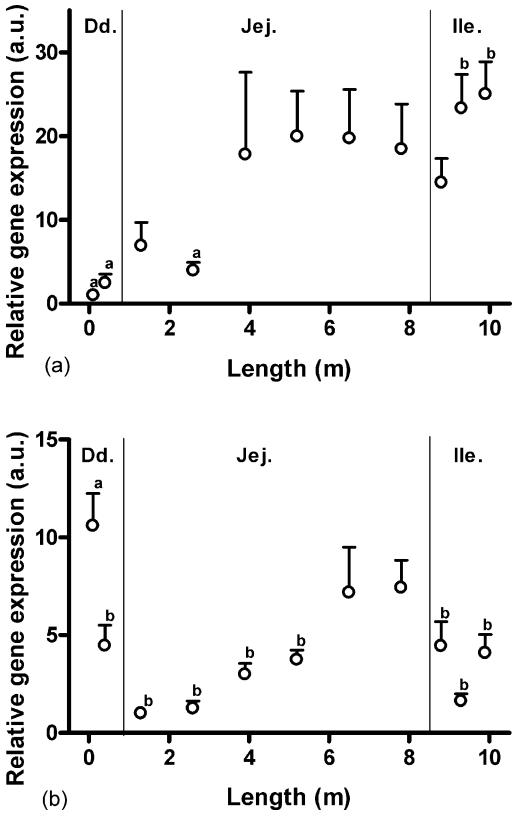

Fig. 2. Relative gene expression of: (a) pBD-1 and (b) pBD-2, in the porcine small intestine. Shown are mean expression values plus S.E.M. Values marked 'a' are significantly different ($p < 0.05$, post hoc Tukey HSD test) from values marked 'b', while non-marked values are not significantly different from any value. Dd, duodenum; Jej, jejunum; Ile, ileum.

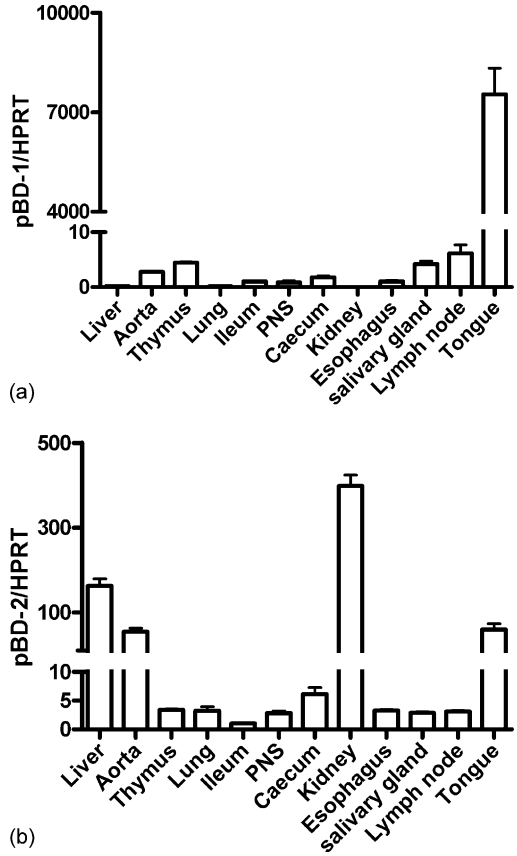

Fig. 3. Tissue distribution of: (a) pBD-1 and (b) pBD-2 mRNA. Values are depicted as relative pBD/HPRT levels plus standard deviation, compared to the jejunum.

3. Results

3.1. Quantitative PCR

Evaluation of quantitative real-time PCR revealed reliable and consistent amplification of the target genes pBD-1, pBD-2 and HPRT. Dilution experiments of RNA aliquots (160 pg–10 ng) resulted in very similar amplification efficiencies of the genes with gradients ranging from −3.1 to −3.4 ($R^2 > 0.95$). Thus, results were normalized against HPRT by calculating the difference in Ct (ΔCt) and expressed as relative expression levels.

3.2. Gene expression of pBD-1 and pBD-2 in the intestine

In order to determine the intestinal gene expression pattern of pBD-1 and pBD-2, mucosal scrapings along the whole intestine were taken from five healthy pigs. Expression of defensins along the intestine was assessed using qPCR and is shown in Fig. 2. For pBD-1, expression is low (close to the detection limit in our system), in the duodenum, and then approximately 10-fold higher and relatively stable along the whole jejunum and ileum (although a small increase from the proximal to distal part can be observed). pBD-2 expression is relatively high in the duodenum, drops at the start of the ileum and then slowly increases further distal along the jejunum which then shows another sudden drop at the beginning of the ileum. Although the differences are not statistically significant ($p > 0.05$) along the jejunum and ileum, this peculiar wave-shaped expression level in the intestine was observed in every single animal.

To relate the intestinal expression of pBDs in the intestine, defensin mRNA levels were also determined in several other tissues and are shown in Fig. 3. The highest mRNA expression of pBD-1 (relative to the household gene HPRT) is observed in the tongue while all other tissues tested showed much lower expression levels. Mucosal scrapings of the tongue even had a 10-fold higher level of pBD-1 mRNA compared to tongue tissues (not shown). For pBD-2 the expression pattern is quite different with kidney, liver and to a lower extend aorta and tongue having the highest mRNA expression. For both defensins it is obvious that, in healthy pigs, starting levels of defensin mRNA in the intestine are relatively low compared to other organs.

3.3. Small intestinal segment perfusion

The morphological characterization of the intestinal samples of the SISP, depicted in Table 2 show some distinct effects. In general, granulocytes are observed after 2 h in all segments, and after 8 h a difference between the number of granulocytes in the lumen of infected and non-infected segments is seen. Granulocytes at other locations in the intestine do not seem to differ

Table 2
Morphological scoring of SISP samples

Time (h)	Segment	Granulocytes				Swelling		
		Lumen	Lymphatic vessels	Serosa	Mesentery	Submucosa	Villi	Abnormal villi
0	**2**	**0.3 (3)**	**0.3 (3)**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	4	**0.0**	**0.0**	**0.0**	**0.2 (2/3)**	**0.0**	**0.0**	**0.0**
	6	**0.1 (3)**	**0.0**	**0.0**	**0.0 (4/4)**	**0.0**	**0.0**	**0.0**
2	1	1.4 (1)	0.5 (2)	1.6 (0)	2.3 (0/4)	0.3 (3)	0.0	0.0
	3	0.4 (2)	1.1 (0)	1.8 (0)	2.5 (0/2)	0.0	0.0	0.0
	5	0.0	0.8 (1)	1.5 (1)	2.0 (0/2)	0.4 (2)	0.0	0.0
	7	0.5 (2)	1.3 (0)	1.8 (0)	2.5 (0/2)	0.3 (3)	0.0	0.0
4	1	1.6 (0)	1.5 (0)	2.8 (0)	2.5 (0/2)	1.3 (3)	0.5 (3)	0.5 (3)
	3	1.6 (0)	2.5 (0)	3.5 (0)	3.8 (0/4)	2.3 (1)	0.0	0.3 (3)
	5	1.3 (1)	2.0 (1)	2.4 (0)	3.0 (0/3)	2.8 (1)	0.8 (2)	1.3 (2)
	7	0.4 (2)	2.5 (0)	3.5 (0)	3.5 (0/2)	2.6 (0)	1.0 (2)	0.5 (2)
8	1	3.5 (0)	3.3 (0)	4.0 (0)	3.0 (0/1)	3.0 (1)	1.3 (2)	1.8 (0)
	2	**0.8 (0)**	**3.0 (0)**	**4.0 (0)**	**3.7 (0/3)**	**1.5 (2)**	**1.3 (2)**	**1.0 (2)**
	3	2.4 (0)	3.0 (0)	4.4 (0)	3.3 (0/3)	4.0 (0)	1.8 (2)	1.3 (2)
	4	**0.6 (1)**	**2.8 (0)**	**3.8 (0)**	**4.0 (0/3)**	**3.3 (0)**	**1.3 (2)**	**1.0 (2)**
	5	1.5 (0)	3.3 (0)	4.4 (0)	4.0 (0/3)	2.0 (0)	0.7 (2)	0.7 (2)
	6	**0.8 (2)**	**3.5 (0)**	**4.3 (0)**	**4.5 (0/4)**	**2.8 (0)**	**1.0 (1)**	**1.5 (0)**
	7	1.9 (0)	3.5 (0)	4.8 (0)	4.5 (0/4)	4.0 (0)	2.0 (1)	1.0 (2)

Values are depicted as the average of segments from four piglets, using a scoring range from 0 (none) to 5 (very abundant) of the characteristic morphology. Between brackets the number of piglets with a score of 0 is depicted. The exception is the column depicting the granulocytes in the mesentery because the mesentery was not always present in the paraffin slides. Here, the average is depicted with the number of zero scores/the number of samples in between brackets. Control (non-infected) segments are shown in bold.

between infected and non-infected segments. Swelling of submucosa starts after 4 h and no difference is observed between infected and non-infected segments after 8 h. Swelling of villi was less distinct but could be observed in a few samples. However, overall morphology of the villi was well preserved after 8 h, indicating that the experimental procedure had a relatively mild effect within the time frame of the experiment on the intestinal morphology.

3.4. Immunohistochemistry

Invasion of *S. typhimurium* was followed by immunostaining using an anti-O *S. typhimurium* antibody. At all time points *S. typhimurium* could be detected in the infected segments, after 2 h mainly in the lumen and epithelium of the villi (Fig. 4), and at later time points also in the lamina propria. Occasionally, *S. typhimurium* was detected in the lymphatic vessels, serosa and mesentery. In a few samples, *S. typhimurium* could also be detected in the serosa and mesentery and lymphatic vessels of the control samples after 8 h. This is consistent with traffic of *S. typhimurium* through the blood or lymphatic system.

3.5. Effect of S. typhimurium on gene expression of pBD-1 and pBD-2

Total RNA was extracted from mucosal scrapings of all intestinal segments and used for quantitative PCR. Expression levels were normalized to the housekeeping gene HPRT and the infected segments were compared to the control segments. The resulting relative expression index for the 8-h infected segments is shown in Fig. 5. Only small differences are observed between control and infected segments with three infected segments having a statistically significantly different expression compared to the control segments ($p < 0.05$): a reduction of pBD-1 mRNA (REI < 1) in the proximal part of the ileum (segment 5), and an increase of pBD-2 in the first part of the jejunum (segment 1) and the distal part of the ileum (segment 7). However, the *S. typhimurium* infected segments taken after 0, 2 or 4 h showed similar defensin mRNA levels compared to the controls (results not shown).

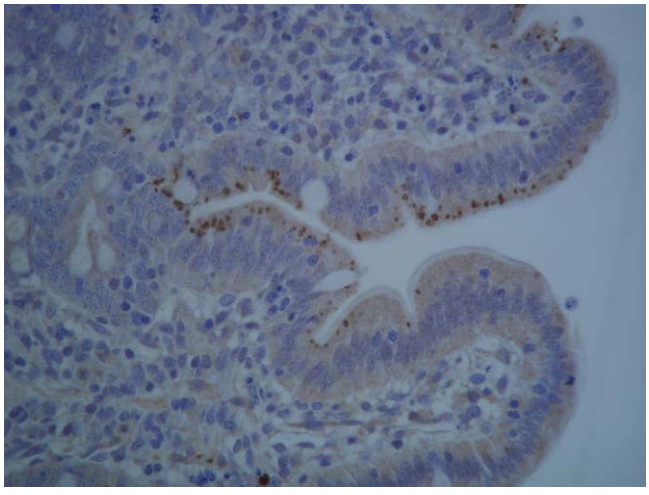

Fig. 4. Detection of *S. typhimurium* by immunohistochemistry. This section shows the proximal ileum (segment 5), 2 h post-infection. *S. typhimurium* was visualized using a specific anti-O *S. enterica* serovar *typhimurium* antibody, biotinylated goat anti-rabbit antibody and finally DAB. Invasion of *S. typhimurium* can be detected in the epithelial layer and the lamina propria.

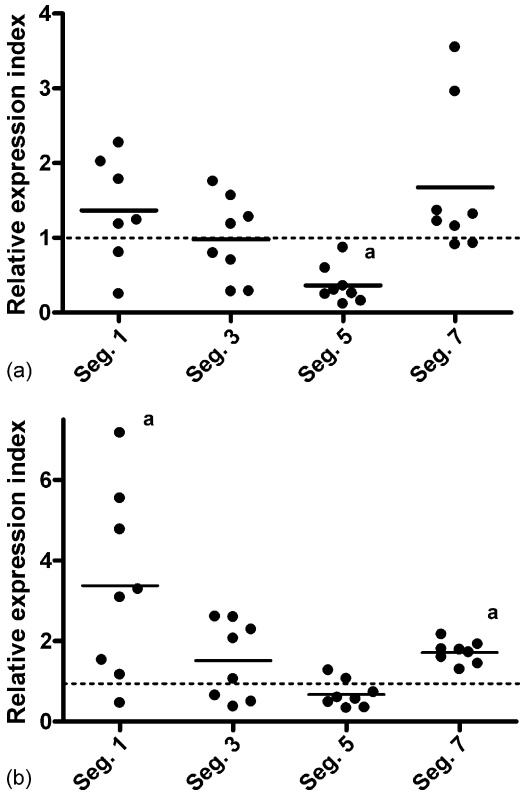

Fig. 5. Relative expression index (REI) of *S. typhimurium* infected segments after 8 h. A REI of 1 is defined as equal expression in the infected segments compared to the average of the control samples. The four infected segments (S1, 3, 5 and 7) are depicted on the *X*-axis while the REI is shown on the *Y*-axis. Infected segments with significantly different ($p < 0.05$, Student's *t*-test) defensin mRNA levels compared to the corresponding control segments are labelled 'a'. (a) pBD-1 and (b) pBD-2.

4. Discussion

In this report, we describe the gene expression pattern of the β-defensins pBD-1 and pBD-2, and the effect of *S. typhimurium* infection on the expression of defensins in the intestinal epithelium, using the small intestinal perfusion technique. The SISP model was originally developed to study the effect of metabolites on water absorption capacity of the intestine (Nabuurs et al., 1993), but has also proven to be a valuable technique for genomic studies (Niewold et al., 2005). In this model, isolated segments of the jejunum and ileum can be infected with a high dose of pathogens while blood supply and innervation remain intact. One of the main advantages of this technique is the minimization of the effect of individual variation between animals because the control segments are within the same animal and in the same part of the intestine as the infected segments. Contrary to oral infections, the technique also provides the possibility to infect the intestinal epithelial cells with a known density of pathogens in vivo. This makes the SISP an important model for gastrointestinal research. In addition, a recently published genome analysis has shown that pig is much closer related to human than rodents (Wernersson et al., 2005). The similarity of especially the alimentary tract also makes the SISP model an important tool for research in human gastrointestinal diseases.

One of our concerns of the SISP model was the effect of extensive handling of intestinal segments during the experiment (where besides the initial operation the segments have to be de- and reattached to the tubes). Therefore, we performed for the first time, an extensive morphological examination of infected and non-infected segments. This showed that, despite some swelling of the submucosa, the overall morphology of the non-infected control segments stayed well preserved (Table 2), during the whole experiment. In addition, quantitative PCR showed that the relative and absolute mRNA levels of defensins did not change (not shown), indicating that the experimental procedure itself had no major impact on expression of the target genes. Furthermore, the epithelial marker I-FAB showed consistent expression in microarray analysis of the mid-jejunum segment (Niewold et al., personal communication) and former studies have shown that the intestinal segments are functionally active with respect to water absorption (Nabuurs et al., 1993), indicating again that no major effect is induced by the experimental procedure. On the later time points in the infection, more granulocytes were observed in both infected and non-infected segments and more swelling of villi and submucosa was visible. However, whether this was an effect due to the experimental procedure or whether it was a systemic reaction to the *S. typhimurium* infection cannot be deduced from the current experimental set-up.

Quantification of defensin mRNA levels was performed by one-step qPCR, a strategy that minimizes experimental variation because both steps (reverse transcription and PCR) are performed in one tube. However, using this technique, it is impossible to detect absolute mRNA levels of the target gene because this would involve comparison to cDNA standards that do not take into account the reverse transcriptase reaction efficiency (Giulietti et al., 2001; Wong and Medrano, 2005). Therefore, relative gene expression compared to the housekeeping gene HPRT was used in this study. Depicting relative expression levels relies on similar PCR amplification efficiencies of the two genes and stable expression of the housekeeping gene among different tissues. Whereas we showed that the amplification efficiency was comparable in our experiments on the intestinal samples, the relative expression of HPRT in different organs was not addressed in our study. However, Oleksiewicz et al. (2001) have shown that in pigs, the expression level of housekeeping genes can vary up to 60-fold among different organs. Therefore, the gene expression levels in Fig. 2 are depicted as relative defensin expression (since all samples are from the intestine) while Fig. 3 depicts the expression levels as relative pBD/HPRT ratios. This indicates that the values depicted in Fig. 3 reflect differences in both housekeeping and target gene and should not be read as absolute expression levels of defensin mRNA. For detection of small differences in expression of the target gene in different organs, multiple housekeeping genes should be measured (Vandesompele et al., 2002), which was outside of the scope of our study. Despite the possible variation in expression levels of the household gene, a general expression pattern in high and low expression sites can be found (Fig. 3). If the differences were merely reflecting HPRT mRNA level differences between tissues, a similar pattern would be observed for both pBD-1 and pBD-2. Obviously, this is not the case. The

expression of pBD-1 correlates well with the pattern described before (Zhang et al., 1998). For pBD-2 no expression sites have been described, but based on these studies, high levels of pBD-2 mRNA are found in liver, kidney and to a lesser extend aorta. One should always keep in mind though that age and health status of the animal can have a major impact on this expression pattern (Caverly et al., 2003; Meyerholz et al., 2004; Stolzenberg et al., 1997).

We hypothesized that the relatively low expression of pBD-1 and pBD-2 in the intestine compared to other tissues could reflect a potential for upregulation of these defensins upon bacterial infection. Regulation of β-defensin levels are, so far, only described at the transcriptional level where many triggers can induce gene expression and subsequent defensin protein levels. However, no large upregulation for either pBD-1 or pBD-2 was observed in our experimental conditions. Even though we found three intestinal segments having statistically significant different gene expression after 8 h (of which one segment showed a downregulation of pBD-1), the in vivo significance of these differences is very doubtful because of the low magnitude of the observed differences and the low starting levels of pBD expression in the intestine. We therefore concluded that there was no true upregulation of pBD-1 or pBD-2 under these experimental conditions. For pBD-1, similar results were described using *S. typhimurium* or well known pro-inflammatory mediators such as LPS, TNF-α and IL-1β in tongue epithelial cell cultures (Liu et al., 2001; Zhang et al., 1999). In addition, analysis of the promoter region of pBD-1 showed no consensus sequences for NF-IL6 or NF-κB, which are present in the inducible EBD and TAP genes (Zhang et al., 1999). The observed lack of induced expression of pBD-1 and pBD-2 could indicate that both defensins are constitutively expressed in the intestine, but it could also mean that other stimulatory factors trigger upregulation. In addition, it is possible that *S. typhimurium* can escape destruction by antimicrobial defence mechanisms by evading the immune response. This might, in fact, be one of the most important mechanisms of *S. typhimurium* to persist in the pig intestine. In this respect, it is interesting to see that this specific *Salmonella* strain only caused a very mild response on gene expression, as deduced by microarray analysis, upon infection of the porcine jejunum (Niewold et al., personal communication), while enterotoxigenic *Escherichia coli*, under the same experimental conditions, caused the upregulation of approximately 300 genes (Niewold et al., 2005). It would be very interesting to test whether other pathogens that cause more symptoms of inflammation, such as the enterotoxigenic *E. coli* would have an effect on pBD expression in the pig intestine.

In summary, gene expression for pBD-1 and pBD-2 was determined and showed a characteristic intestinal expression pattern for both defensins. The highest expression levels were found in tongue (pBD-1) and kidney and liver (pBD-2). Small segment intestinal perfusion of piglets was used to test whether *S. typhimurium* DT104 could upregulate porcine β-defensins 1 and 2 in vivo. No effect on pBD levels could be observed, indicating that intestinal pBD expression is either constitutive, or that *S. typhimurium* is capable of evading the host innate immune response.

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