UNIVERSITEIT GENT

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IMPORTANCE OF HEAT-STABLE ENTEROTOXIN B IN THE INDUCTION OF EARLY IMMUNE RESPONSES IN PIGLETS AFTER INFECTION WITH ENTEROTOXIGENIC *ESCHERICHIA COLI*

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INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) are a major cause of dehydrating diarrhoea in children and weaned piglets living under subhygienic conditions. After colonization of the small intestine, ETEC produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. However, the relative importance of the different enterotoxins in the pathogenesis of ETEC infection has been poorly defined.

OBJECTIVES

We wanted to assess the **contributions of the different enterotoxins** of an ETEC strain to the induction of small intestinal secretion and early innate immune responses in weaned piglets. **Isogenic mutant strains** of an LT$^+$ STa$^+$ STb$^+$ ETEC strain were constructed that lack the expression of LT in combination with one or both types of ST enterotoxins (STa and/or STb). The small intestinal segment perfusion technique and microarray analysis were used to study **porcine early immune responses** induced by these mutant strains 4h after infection in comparison to the wild type strain and a PBS control. Simultaneously, **net fluid absorption** of pig small intestinal mucosa was measured **4h after infection**.

MATERIALS AND METHODS

Bacterial strain and mutants

Enterotoxin deletion mutants of the hemolytic porcine E. coli strain GIS26, serotype O149:K91:F4ac were generated using the bacteriophage lambda recombinase system (λ-Red) of Datsenko and Wanner [1]. Enterotoxin production was quantified in the different mutant strains using specific enzyme immunoassays. The enterotoxin phenotype of the used strains is shown in **Table 1**.

Genotype	Phenotype STa	STb	LT	Strain designation
wild type	+	+	+	**GIS26(STa$^+$ STb$^+$ LT$^+$)**
ΔeltAB	-	+	-	**GIS26(STa$^-$ STb$^+$ LT$^-$)**
ΔestBΔeltAB	+	-	-	**GIS26(STa$^+$ STb$^-$ LT$^-$)**
ΔestA	-	Low	-	**GIS26(STa$^-$ STb^{low} LT$^-$)**
ΔestAΔestB:KAN	-	-	-	**GIS26(STa$^-$ STb$^-$ LT$^-$)**

Table 1 Enterotoxin phenotype of GIS26 mutants used in this study

Small Intestinal Segment Perfusion

For this study we used three 5-week-old weaned piglets. The abdomen of anesthetized piglets was opened and 6 segments of 20 cm length were made in the mid jejunum. The segments were cannulated with silicone tubes at the proximal and distal ends (**Figure 1**) to inject and collect fluid respectively .

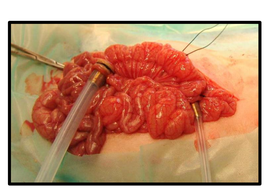

Figure 1 Cannulated segment

Segments were first injected with 2,5x10^9 CFU of wild type GIS26, one of the four GIS26 mutant strains or with PBS only. Each segment was then perfused during 4h by injecting 2ml perfusion buffer every 15 minutes. At the end of the experiment net fluid absorption of each segment was calculated from the difference between inflow and outflow divided by the surface area of each segment. A small piece of tissue of each segment was sampled for RNA isolation

Microarray Analysis

We used the Porcine Genome Array (Affymetrix) containing 23,937 probe sets, representing 20,201 Sus scrofa genes.

The normalized intensity values of the different conditions were compared and differential transcripts were selected based on the more stringent cut-off of the uncorrected P-values , i.e. P<0.001. This cut-off on the P-values was combined with a cut-off on the fold-change of two (i.e. an absolute log2-ratio > 1).

qRT-PCR:

Nine genes from the microarray analysis *(IL8, PAP, FABP2, IL1A, IL17A, TLR4, MMP1, MMP3* and *CYP1A1*) were selected for confirmation by quantitative real-time PCR. The relationship between the levels of gene expression of these genes (qRT-PCR versus microarray data) was determined by linear regression (data not shown).

[1] Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640-6645.

RESULTS AND DISCUSSION

STb seems to play an important role in the induction of intestinal secretion

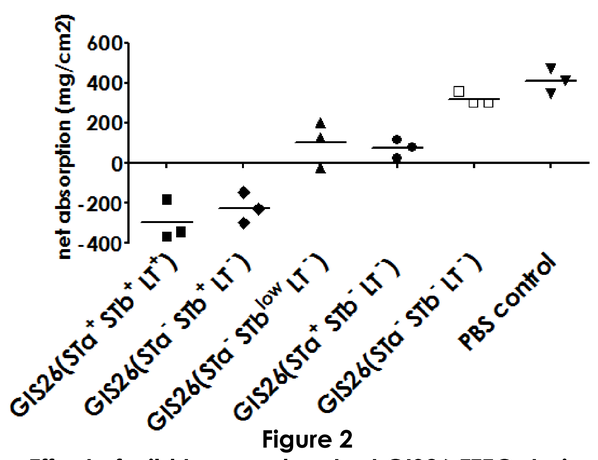

Figure 2
Effect of wild type and mutant GIS26 ETEC strains on net fluid absorption (mg/cm2) in 4h-infected jejunal segments

Small intestinal segment perfusion with the different strains (**Figure 2**) showed:

- ✓ no difference in intestinal secretion induced by the wild type strain and the mutant only expressing STb.
- ✓ a significant difference between the STb$^+$ and STb^{low} strain (P<0,01) indicating that the amount of STb produced is important.
- ✓ that the strain secreting only STa induced a significant lower effect on net absorption compared to the wild type (P<0,01), indicating that STa only plays a minor role.
- ✓ that the mutant strain not expressing enterotoxins is no longer able to reduce net absorption.

ETEC regulates expression of porcine genes important in inflammation

- ✓ PBS versus ETEC: 153 transcripts down-regulated and 157 transcripts up-regulated
- ✓ 15 transcripts are down-regulated by ETEC with log-ratio < -2 and these transcripts are associated with the intestinal metabolism or with transport of fluids and electrolytes.
- ✓ 23 transcripts are up-regulated by ETEC with log-ratio>2 and the 13 of them represent immunomodulatory genes (**Table 2**).

Gene	Gene symbol	Probe Set ID	Log2 ratio
Matrix metalloproteinase 3	*MMP3*	Ssc.15927.1.S1_at	**4.18**
		Ssc.15927.2.S1_at	**4.16**
		Ssc.15927.2.A1_at	**2.90**
Interleukin-17A	*IL17A*	SscAffx.23.1.S1_at	**3.68**
Pancreatitis associated protein	*PAP (REG3A)*	Ssc.16470.1.S1_a_at	**3.10**
Interleukin-1 beta	*IL1B*	Ssc.17573.1.S1_at	**2.72**
		Ssc.15601.1.A1_s_at	**2.65**
Interleukin-1 alpha	*IL1A*	Ssc.113.1.S2_at	**2.68**
		Ssc.113.1.S1_at	**2.30**
Dual oxidase 2	*DUOX2*	Ssc.33.1.S1_at	**2.33**
Matrix metalloproteinase 1	*MMP1*	Ssc.16013.1.S1_at	**2.05**
Ectoderm-neural cortex protein 1	*ENC1*	Ssc.30857.1.S1_at	**2.05**
interleukin-1 receptor antagonist	*IL1RN*	Ssc.16250.1.S2_at	**2.01**

Table 2 Transcripts of immune related genes upregulated by wild type ETEC

Microarray results suggest a role for STb in ETEC-induced immune responses

- ✓ Only 2 mutant strains showed differential expression from wild type (**Figure 3**).
- ✓ 15 transcripts are up- or down-regulated by ETEC but not related to enterotoxin expression. We suggest a regulation of these genes by LPS or other metabolites. Among them are *MMP1, PAP, IL8, IL1RN* and *DUOX2*.
- ✓ 26 transcripts are differentially regulated by the 3 strains secreting normal levels of STb and/or STa. Among them are *MMP3* and *IL1A*.
- ✓ 24 transcripts are still differentially regulated by the Stb^{low} strain but not by the enterotoxin negative strain, indicating a role for STb in their regulation. Among them are *IL17A* and *IL1B*.

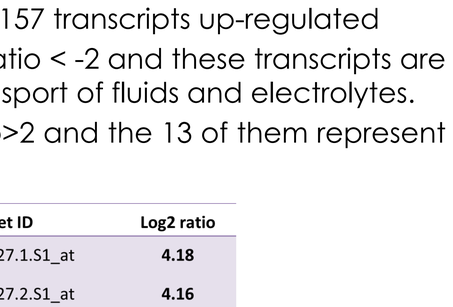

Figure 3
Number of differential regulated transcripts for the comparisons made

CONCLUSION

Microarray analysis showed on the one hand a **non-toxin related general antibacterial response** comprising genes such as pancreatitis-associated protein, interleukin 8 and matrix metalloproteinase 1. On the other hand, results demonstrated an **important role for STb in small intestinal secretion** early after infection **as well as in the ETEC induced immune response** by the significant differential regulation of immune mediators like matrix metalloproteinase 3, interleukin 1 and interleukin 17.