# The Impact of *ymoA* Gene Expression on Enterotoxin YstA Production by *Yersinia Enterocolitica* Strains With Different Enterotoxic Properties

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# Abstract

*Yersinia enterocolitica* is one of the main causative agents of human diarrhoea and the reservoir and source of infection for humans are pigs. Strains isolated from humans with clinical yersiniosis and diarrhoea are able to produce *Yersinia* stable toxins – Yst. However, enterotoxin-producing capabilities have been attributed to the *ymoA* gene which encodes the production of the *Yersinia* modulator protein – YmoA. The aim of this study was to analyse *ystA* and *ymoA* genes expression in *Y. enterocolitica* strains with different enterotoxic properties, isolated from humans and pigs. The experiment involved two groups of *Y. enterocolitica* strains producing and not producing enterotoxin YstA, which were isolated from humans and pigs. All these strains were *ystA*- and *ymoA*-positive. The relative expression level of the *ystA* gene was significantly higher than the expression level of the *ymoA* gene in *Y. enterocolitica* strains isolated from humans with clinical signs characteristic for yersiniosis. In others, a significant decrease in *ystA* gene transcription was observed, and the relative expression level of the *ymoA* gene was significantly higher than the expression level of the *ystA* gene. Statistically significant differences were not observed in either group of strains isolated from pigs. The results of our study revealed a correlation between the mRNA expression levels of *ystA* and *ymoA* genes in *Y. enterocolitica* strains isolated from humans.


# Background

*Yersinia enterocolitica* is one of the main causative agents of human diarrhoea with growing epidemiological importance[1]. Various authors have postulated a correlation between strains isolated from healthy pigs and yersiniosis in humans[2-5]. These findings suggest that pigs are the main reservoir and source of infection for humans. Strains isolated from humans with clinical yersiniosis and diarrhoea are able to produce enterotoxins *Yersinia* stable toxins (Yst), which indicates that Yst play a significant role in the aetiology of diarrhoea that accompanies the disease and is one of the key virulence factors of *Y. enterocolitica*. Two main groups of *Y. enterocolitica* enterotoxins have been identified: enterotoxin YstI which includes variants YstA, YstB, and YstC, and enterotoxin YstII whose mechanism of action probably differs from that of YstI[6].

The best-known *Y. enterocolitica* enterotoxin, YstA, is a 30-amino-acid peptide whose mechanism of action is based on activation of guanylate cyclase. This mechanism of action is highly similar to that found in enterotoxin STI produced by *Escherichiacoli,* and it is responsible for an increase in cGMP level in intestinal epithelial cells and extracellular accumulation of liquid[7-8]. The opponents of the hypothesis postulating that YstA is the main cause of diarrhea during yersiniosis have pointed out that YstA is not produced at temperatures higher than 30°C. They have argued that enterotoxin YstA is unlikely to induce diarrhoea since the temperature in the intestines approximates 37°C. However, Mikulskis et al.[9] demonstrated that *ystA* transcription can be induced at 37°C by providing the pH of the culturing similar to that in the ileum at pH 7.5. They showed that under such conditions, YstA production is identical to that noted at temperatures below 30°C. Enterotoxins YstB and YstC are produced by *Y. enterocolitica*

strains belonging to biotype 1A. These strains are generally considered as non-pathogenic, however recent research indicates that they could play a role in diarrhoea induction[10].

YstA is encoded by the *ystA* gene, but not all *ystA*-positive strains produce enterotoxin. Enterotoxin-producing capabilities have been attributed to the *ymoA* gene which encodes the production of the Yersinia modulator (YmoA) protein. YmoA belongs to the family of nucleoid-associated proteins and its sequence is in 82% identical with the regulator of high haemolysin activity (Hha) proteins in *E. coli* and *Salmonella*[11]. YmoA influences DNA supercoiling and forms heterodimers with histone-like nucleoid structuring (H-NS) proteins[12-15]. H-NS play important roles as structural proteins and gene expression modulators[16-17]. Peruzy et al.[18] recently showed that 161 *Y. enterocolitica* strains of differed origins, tested for the *ymoA* gene presence, were positive. It means that to evaluate the role of *YmoA* as another genes modulator it's expression should be examined. The expression of genes responsible for the pathogenicity of *Y. enterocolitica* has been broadly investigated. Research results indicate that YmoA is one of the main modulators of gene expression in response to environmental factors[19-20] and that it participates in the negative regulation of virulence marker transcription[21-22].

The possible influence of *ymoA* on *yst* genes was first postulated by *Cornelis et al.*[21] *who* insinuated that the *ymoA* mutation unblocks the silencing of the *yst* gene and stimulates enterotoxin production. However, the results of our previous study[23] show that two point mutations in the nucleotide sequence of the *ymoA* gene, which were detected with the use of the High-Resolution Melting (HRM) method, did not influence on enterotoxic properties of the examined strains. Mikulskis et al.[9] in 1994 presented the mechanism modifying the expression of *yst* to a silent state. According to the cited authors, gene silencing was caused by modifications in the status of bacterial host factors, and YmoA participated in both *yst* silencing and temperature regulation. YmoA was identified as one of the factors necessary for growth-phase regulation of *yst*. In 1998, Grant et al.[24] have also suggested that the lack of enterotoxic properties in selected *Y. enterocolitica* strains could result from the inhibitory influence of the *ymoA* gene on *ystA* gene expression in *in vitro* cultures. To date, this possibility has been investigated only by Starke and Fuchs[25] who identified YmoA as a silencing factor for all toxic complex (*tc*) genes of *Y. enterocolitica* strain W22703 (biotype 2, serotype O:9).

The purpose of the study was to analyse *ymoA* gene expression in *Y. enterocolitica* strains with different enterotoxic properties and to evaluate the inhibitory effect of the *ymoA* gene on the production of enterotoxin YstA by the strains isolated from humans and pigs.

## Materials And Methods

# Materials

This study was performed retrospectively, based only on bacterial strains and did not require ethical approval. *Y. enterocolitica* strains were previously isolated from samples routinely submitted to the

diagnostic laboratories and obtained from infected humans. *Y. enterocolitica* strains isolated from pigs were obtained from a previous study[26]. The experimental material consisted of 74 *Y. enterocolitica* strains isolated from humans and 51 *Y. enterocolitica* strains isolated from pigs. In this study, only *Y. enterocolitica* strains from infected humans and animals were analysed, therefore, control groups (uninfected) could not be established.

## *Y. enterocolitica* strains isolated from humans

Group I was composed of 34 *Y. enterocolitica* strains isolated from clinical cases of yersiniosis (anonymous data from medical history delivered by laboratories). Group II consisted of 40 *Y. enterocolitica* strains isolated from humans with unknown clinical diagnosis. All *Y. enterocolitica* strains were before biotyped, serotyped and molecularly examined (*ystA, ystB, ystC, ymoA*). Primer sequences and PCR conditions were described previously[26]. Group I consisted of 13 *Y. enterocolitica* strains belonging to a rare in Poland bioserotype 1B/O:8 and 21 strains belonging to bioserotype 4/O:3. Group II consisted of *Y. enterocolitica* strains belonging only to bioserotype 4/O:3 because highly pathogenic bioserotype 1B/O:8 strains have never been isolated from humans without clinical yersiniosis. All *Y. enterocolitica* strains used in this study were *ystA*- and *ymoA*-positive.

## *Y. enterocolitica* strains isolated from pigs

Fifty-one *Y. enterocolitica* strains isolated from fattening pigs without clinical signs of yersiniosis were examined. The enterotoxic properties of these strains were determined previously, using suckling mouse bioassay[26]. Enterotoxin production was evaluated by measuring the ratio of intestinal mass to the remaining body mass in three examined sucklings. According to Gianella[27], a ratio of $\leq 0.074$ indicates a negative result, a ratio of 0.075–0.082 denotes a doubtful result, and a ratio of $\geq 0.083$ represents a positive result. In this study, 13 *Y. enterocolitica* strains producing enterotoxin YstA in the suckling mouse bioassay formed Group I, and 38 *Y. enterocolitica* strains not producing enterotoxins in the suckling mouse bioassay formed Group II. All examined strains belonged to bioserotype 4/O:3 and were *ystA*- and *ymoA*-positive.

## RNA preparation and reverse transcription

Bacteria were grown in tryptic soy broth (TSB) at 28°C, and the inoculated medium was incubated with shaking (250 r.p.m.) by 24h. The cells were harvested by centrifugation in an Eppendorf Centrifuge 5804 R for 5 min. at a speed of 3100xg and the supernatant was discarded. Total RNA extraction was done with cell pellets containing $1 \times 10^7$ cells using RLT Buffer, being a part of RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany). This kit includes the RNAprotect Bacteria Reagent for stabilizing RNA in bacterial samples and RNeasy spin columns for purifying up to 100 µg of high-quality RNA using the silica-membrane technology. Next steps of RNA extraction were done according to the manufacturer's instructions. RNA integrity was assessed by agarose gel electrophoresis. RNA concentration and quality

were measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). An $A_{260}/A_{280}$ ratio of 2.0 (in the range of 2.06–2.13) was considered pure RNA. Reverse transcription (RT) into cDNA was carried out with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was stored at -20°C until further use.

# Gene expression analysis using qPCR

Selected genes were analysed by quantitative real-time PCR (qPCR) with the Rotor-Gene6000™ real-time analyser (Corbett Life Science, Sydney, Australia). The expression of *ystA* and *ymoA* was normalized to that of the *gapA* and *polA* reference genes, encoding D-glyceraldehyde-3-phosphate dehydrogenase, and production of DNA polymerase I, respectively[28]. The forward and reverse primers used in this study are shown in Table 1. Every sample for *ystA*, *ymoA*, and *gapA, polA* mRNA analysis contained cDNA (70 ng), forward and reverse primers (final concentration of 0.7 µM/l each) and the QuantiTect SYBR Green RT-PCR master mix (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Standard curves of serial dilutions of the appropriate purified cDNA were used for quantification. Each PCR reaction (25 µl) was performed in duplicate in a 36-well rotor under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing at 52°C for 30 s, followed by elongation at 72°C for 45 s. Final elongation at 72°C for 10 min was carried out for each PCR reaction. Melting curves were obtained based on stepwise increase in the temperature ramp from 65°C to 90°C to ensure the amplification of a single product for each reaction.

Table 1
Sequences of the primers used in the study

| Gene | Forward primer | Reverse primer | Reference |
|---|---|---|---|
| *ystA* | 5'GTCTTCATTTGGAGGATTCGGC3' | 5'AATCACTACTGACTTCGGCTGG3' | Platt-Samoraj et al.[29] |
| *ymoA* | 5'GACTTTTCTCAGGGGAATAC3' | 5'GCTCAACGTTGTGTGTCT3' | Grant et al.[24] |
| *polA* | 5'-GCTGGCTTGCGGATGTAGAT-3' | 5'-AGCACGGCGGTCACTTCA-3' | Townsend et al.[30] |
| *gapA* | 5'-CCATCCGTGTTACCGCAGAG-3' | 5'-TCTTAGCACCAGCAGCAATGT-3' | Townsend et al.[30] |

# Data analysis and statistical analysis

Amplification curves were generated from real-time qPCR data. The cycle threshold (CT) was calculated based on a fluorescence threshold of 0.01 and specified as a cycle at which an amplified product was first detected. Then the ΔCT for each sample was established using the equation *ΔCT = CT target gene—CT reference gene* to calculate the expression of each gene relative to the internal reference control. This

was accomplished by modifying the original equation to the relative expression of 2 − ΔCt for the samples[31–32]. All statistical analyses were performed in the Graph-Pad PRISM v. 6.0 programme (GraphPad Software, Inc., San Diego, CA, USA). To test the expression of *ystA* and *ymoA* mRNA in different groups of *Y. enterocolitica* strains, two-way ANOVA was performed. All numerical data are expressed as means ± SEM at a significance level of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

# Results

*In vitro* and *in vivo* expression of bacterial virulence factors can be examined with the use of reference strains which are subject to observable change when genes of interest are expressed[33]. To understand *in vitro* expression and the possible role of the *ymoA* gene during diarrhoea induction, we monitored the expression of *ystA* and *ymoA* genes in *Y. enterocolitica* strains with known enterotoxic properties, observed *in vivo*. All examined strains were *ystA*-, and *ymoA*-positive, irrespective of their ability to produce enterotoxin in vivo. *Y. enterocolitica* strains isolated from humans with clinical yersiniosis and *Y. enterocolitica* strains isolated from pigs and capable of producing enterotoxin YstA in the suckling mouse bioassay were used in this experiment. To compare, *Y. enterocolitica* strains isolated from infected humans with unknown clinical diagnosis and *Y. enterocolitica* strains isolated from pigs and not producing the enterotoxin in the suckling mice bioassay, were used.

The relative expression level of the *ystA* gene was significantly higher ($p < 0.001$) than the expression level of the *ymoA* gene in *Y. enterocolitica* strains isolated from humans with clinical yersiniosis (Group I). The reverse was noted in the group of *Y. enterocolitica* strains isolated from humans with unknown clinical diagnosis (Group II) – a significant decrease in *ystA* gene transcription was observed in all these strains, and the relative expression level of the *ymoA* gene was significantly higher ($p < 0.05$) than expression level of the *ystA* gene (Fig. 1a). Therefore, correlation was found between the relative levels of *ystA* and *ymoA* mRNA.

Statistically significant differences were not observed in either group of strains isolated from pigs. Significant differences in the relative expression levels of *ystA* and *ymoA* genes were not noted in *Y. enterocolitica* strains isolated from pigs and capable of producing enterotoxin YstA in the suckling mouse bioassay (Group I). In the group of *Y. enterocolitica* strains isolated from pigs and not capable of producing enterotoxin YstA (Group II), the relative expression level of the *ymoA* gene was higher than the expression level of the *ystA* gene, but the observed differences were not statistically significant (Fig. 1b). Therefore, no correlation was found between the relative levels of *ystA* and *ymoA* mRNA, as was observed in humans.

The results of a statistical analysis of *ystA* and *ymoA* mRNA levels in *Y. enterocolitica* strains isolated from humans with clinical yersiniosis revealed minor differences between bioserotypes. The significance of the observed differences was determined at $p < 0.001$ in *Y. enterocolitica* strains belonging to the highly pathogenic bioserotype 1B/O:8 (Fig. 2a) and at $p < 0.05$ in *Y. enterocolitica* strains belonging to bioserotype 4/O:3 (Fig. 2b). Since highly pathogenic strains of bioserotype 1B/O:8 were not isolated from

humans without clinical yersiniosis, Group II strains of *Y. enterocolitica* belonging only to bioserotype 4/O:3 were used for comparison. Similar statistical trends in the mRNA expression levels of *ystA* and *ymoA* genes in *Y. enterocolitica* strains were noted in Group II in comparison to both Groups I.

To summarize, the same *ystA* gene was expressed differently in the tested groups of *Y. enterocolitica* strains isolated from humans. Based on the relative expression levels of *ystA* and *ymoA* genes the strains represented two different groups. The strains obtained from patients with clinical yersiniosis expressed the *ystA* gene, whereas in *Y. enterocolitica* strains isolated from humans with unknown clinical diagnosis the expression of the *ystA* gene was lower than *ymoA.* In both groups of *Y. enterocolitica* strains, the expression level of *ystA* mRNA was correlated with the expression level of *ymoA* mRNA. Statistically significant differences were not observed in either group of strains isolated from pigs.

# Discussion

It has been long suggested that YmoA is an important determinant of the production of enterotoxin Yst by *Y. enterocolitica* strains[9,21,24]. Research aiming to confirm or rule out the above hypothesis has not been undertaken since the above observation had been made. Although, YmoA has been confirmed as a negative regulator of the transcription of other virulence markers, such as *inv*, which encodes invasin – the essential factor of internalization, responsible for the transport of *Y. enterocolitica* across M cells[21–22]. YmoA was also shown to participate in production of *Yersinia* outer proteins (Yops) and *Yersinia* adhesin (YadA), dependent on temperature[22]. More recently, Böhme et al.[11] described YmoA as a thermo-sensitive virulence modulator protein which optimizes temperature apperception and fine-tunes virulence gene expression during infection. To better understand the regulatory factors that contribute to enterotoxin production by *Y. enterocolitica*, we have examined the molecular mechanism that switches *ystA* expression to a silent state. Our recent study revealed that two point mutations in the coding region of the *ymoA* gene nucleotide sequence do not affect the enterotoxic properties of the examined strains[23]. Our findings did not confirm the postulated influence of *ymoA* mutations on *ystA* gene silencing [9,21]. However, analyses of genes encoding H-NS proteins in *Yersinia* spp. are hampered by the fact that their mutations are harmful for cells[34,35]. Our study was prompted by the above observation as well as the hypothesis that decreased expression of the *ystA* gene in *in vitro* cultures could be responsible for the absence of enterotoxic properties in selected *Y. enterocolitica* strains.

Results obtained in this study broaden the knowledge about interactions between *ystA* and *ymoA*, including their involvement in the pathogenicity of *Y. enterocolitica*, and the spectrum of virulence genes that are controlled by YmoA. We observed a significant reduction of *ystA* gene transcription in strains isolated from humans with unknown clinical diagnosis. The relative expression level of the *ymoA* gene was significantly higher than the expression level of the *ystA* gene. In patients diagnosed with yersiniosis, the relative expression level of the *ymoA* gene was significantly lower than the expression level of the *ystA* gene. The above was particularly evident in *Y. enterocolitica* strains belonged to the highly pathogenic bioserotype 1B/O:8, responsible for the most severe cases of the disease. Differences were

also observed in the mRNA expression of the *ystA* gene in *Y. enterocolitica* strains isolated from humans with yersiniosis and belonged to bioserotype 4/O:3, but they were less significant than those noted in bioserotype 1B/O:8 strains.

According to our knowledge, this study is the second research attempt to investigate the influence of YmoA on the production of enterotoxins by *Y. enterocolitica*. The other study was conducted by Starke and Fuchs[25] who demonstrated that YmoA silenced all *tc* genes of *Y. enterocolitica* strain W22703 (biotype 2, serotype O:9). Using fusions of promoter with the luciferase reporter, they detected that the deletion of *ymoA* increased the transcription of *tcaR1*, *tcaR2*, *tcaA*, *tcaB*, *tcaC*, *tccC1* and *tccC2* at 15°C and 37°C temperatures. They also observed that at low temperatures, the amount of thermostable YmoA in cells was not reduced, but the repressor was less functional. In the cited study, supplementation by episomal *ymoA* greatly reduced *tc* gene expression, thus confirming the inhibitory influence of YmoA on the production of insecticidal proteins. According to Starke and Fuchs[25], YmoA facilitates H-NS binding to *tc* promoters by creating a compound with this nucleoid-associated protein. The resulting compound not only binds to the upstream regions of all *tc* genes, but also to intragenic sites of *tcaA* and *tcaB;* therefore, it plays a significant role due to control the expression of both genes. Those observations are in line with our findings, which indicate correlation between *ystA* and *ymoA* expression levels.

However, further research involving *ymoA* mutants seems to be essential to validate this observation as the similar differences were not found in *Y. enterocolitica* strains isolated from pigs. Interestingly, a decrease in the mRNA expression of the *ymoA* gene was not observed in *Y. enterocolitica* strains isolated from pigs and producing enterotoxin YstA in the suckling mouse bioassay. The above could be attributed to the fact that we disposed only 13 such strains. The relative expression level of the *ymoA* gene was higher than the expression level of the *ystA* gene in the group of *Y. enterocolitica* strains which were isolated from pigs and not able to produce enterotoxins, but the noted differences were not statistically significant. The use of more strains with proven ability to YstA enterotoxin production may allow the statistically significant results obtain. An interesting aspect would also be an examination of *Y. enterocolitica* strains isolated from other animal species and from food. If the correlation between *ystA* and *ymoA* genes expression levels would not be confirm in a larger number of toxin-producing strains, this could indicate existence of the factors that co-operate with YmoA in *Y. enterocolitica* strains isolated from humans.

# Conclusions

The results of our study revealed a correlation between the mRNA expression levels of *ystA* and *ymoA* genes in *Y. enterocolitica* strains isolated from humans. However, given the lack of statistically significant differences between *Y. enterocolitica* strains isolated from pigs and characterized by different enterotoxic properties, further analyses involving a larger number of *Y. enterocolitica* strains and *ymoA* mutants are needed to confirm this observation.

# Declarations

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### Author Contributions

A.B K conceived, designed and performed the experiments, analysed the data and wrote the paper; K.LI performed the experiments.

### Competing interests

The authors declare no conflict of interest.

### Availability of data and materials

The data generated and/or analyzed during the current study are available from the corresponding authors upon request.

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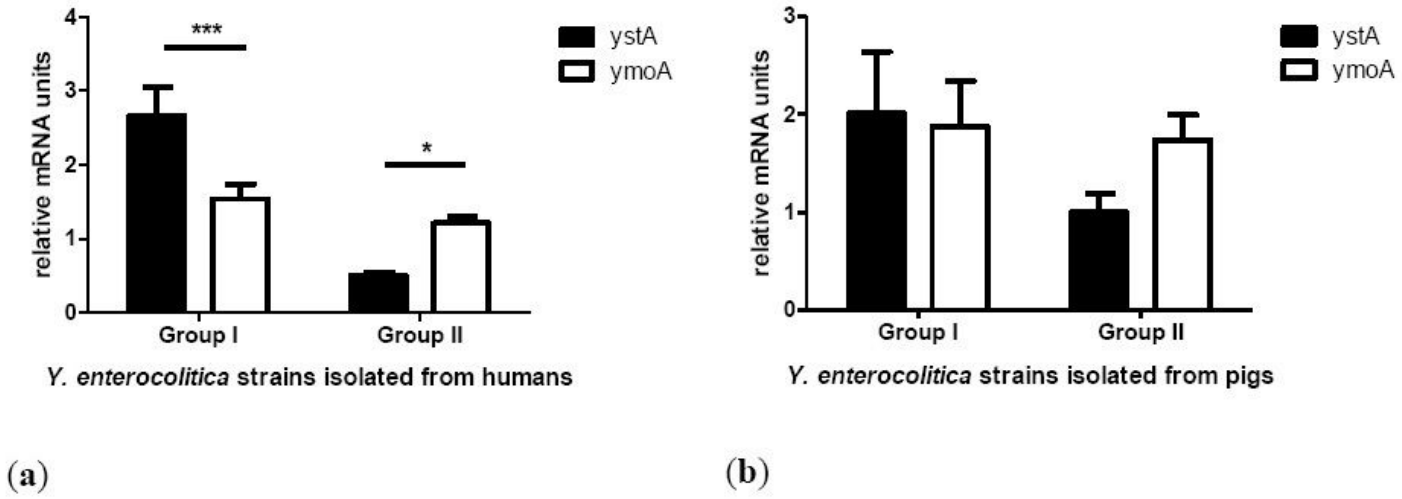
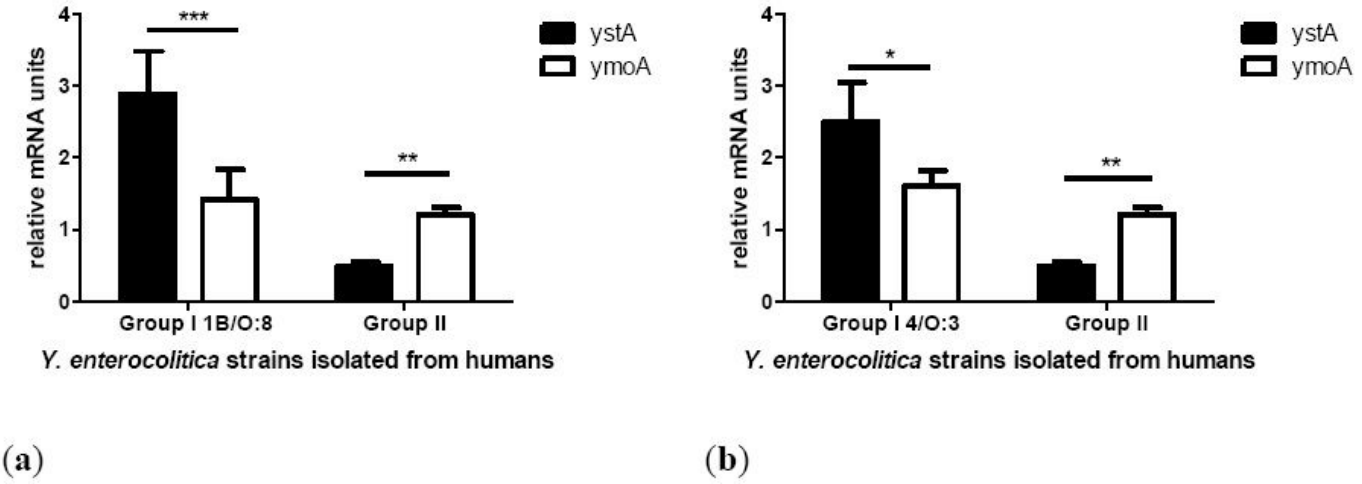
# Figures




## Figure 1

The expression of ystA and ymoA mRNA in Y. enterocolitica strains with different enterotoxic properties, isolated from humans and pigs. The results of real-time PCR for ystA and ymoA genes were normalized against the expression of gapA and polA genes. Data are expressed as the mean ±SEM, and asterisks indicate differences between groups (*p<0.05, ***p<0.001). (a) Y. enterocolitica strains isolated from humans; Group I consists of strains isolated from humans with clinical yersiniosis; Group II consists of strains isolated from humans with unknown clinical diagnosis (b) Y. enterocolitica strains isolated from pigs; Group I consists of strains capable of producing enterotoxin YstA in the suckling mouse bioassay; Group II consists of strains unable to produce enterotoxin.




## Figure 2

The expression of ystA and ymoA mRNA in Y. enterocolitica strains with different bioserotypes, isolated from humans. The results of real-time PCR for ystA and ymoA genes were normalized against the expression of gapA and polA genes. Data are expressed as the mean ±SEM, and asterisks indicate differences between groups (*p<0.05, **p<0.01, ***p<0.001). (a) Group I consists of Y. enterocolitica strains belonging to bioserotype 1B/O:8, isolated from humans with clinical yersiniosis; Group II consists of strains isolated from humans with unknown clinical diagnosis; (b) Group I consists of Y. enterocolitica strains belonging to bioserotype 4/O:3, isolated from humans with clinical yersiniosis; Group II consists of strains isolated from humans with unknown clinical diagnosis.