

Distribution of the *ymoA* and *ystA* genes and enterotoxins Yst production by *Yersinia enterocolitica* strains isolated from humans and pigs

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Abstract

Yersinia (Y.) enterocolitica is the third etiological agent of human diarrhea in terms of the number of confirmed clinical cases. One of the important virulence markers is the *yst* gene which encodes the production of enterotoxins Yst (*Yersinia* stable toxins). However, not all strains with *yst* genes produce enterotoxins, what seems to be caused by the *ymoA* gene encoding the production of the YmoA protein inhibiting the expression of various genes. The purpose of our study was to evaluate the distribution of the *ymoA* and *ystA* genes and Yst production by *Y. enterocolitica* isolated from humans and pigs. All the studied strains obtained from pigs had the *ystA* gene which indicates that they belong to the group of strains commonly regarded as pathogenic, but the ability to produce YstA was detected in only 14 out of 96 examined strains. The fragments of *ystA* gene were also detected in all *Y. enterocolitica* strains isolated from human cases of diarrhea. Amplification of a fragment of the *ymoA* gene was detected in all the studied strains, both from humans and pigs, based on the presence of a 330 bp band. Thus no correlation was identified between the occurrence of the *ymoA* and *ystA* genes and the production of a specific type of enterotoxin.

Key words: *Y. enterocolitica*, enterotoxins Yst, YmoA protein, multiplex PCR, suckling mouse bioassay

Introduction

Yersinia (Y.) enterocolitica is the third aetiological agent of human diarrhea in terms of the number of confirmed clinical cases, after *Campylobacter spp.* and *Salmonella spp.* (Osek et al. 2011). The correlation between strains isolated from pigs and those which evoke clinical signs in humans has been sufficiently proven, justifying selection of this animal species as the study object (Boghenbor et al. 2006, Kelesidis

et al. 2008, Farzan et al. 2009, Laukkanen et al. 2009). Due to the symptomatic diversity of the disease, it is extremely difficult to recognise it by the clinical picture alone, both in humans and in animals. Laboratory tests necessary for proper diagnosis are time- and labour-consuming. Therefore, molecular methods involving detection of virulence markers are more frequently applied. One of the most important virulence markers is the *yst* gene which encodes enterotoxins Yst (*Yersinia* stable toxins) production.

Yst is a 30-amino-acid peptide whose properties are very similar to those of STI (a heat-stable toxin type I) produced by *Escherichia (E.) coli*. They have the same mechanism of action, based on guanylate cyclase activation resulting in the increased cGMP levels in enterocytes and extracellular accumulation of liquid in the intestine (Delor et al. 1990, Revell et al. 2001). Opponents of the theory indicating Yst as the key factor of diarrhea induction in the course of yersiniosis have pointed the lack of Yst production in a temperature over 30°C and the biological activity of those environmental strains commonly regarded as non-pathogenic. Taking into account that the temperature in the intestines is around 37°C, the role of the enterotoxin Yst in diarrhea induction has seemed doubtful. However, the studies of Singh et al. (2004) showed that the ability to produce Yst at a temperature of 37°C in a slightly alkaline environment (pH 7.5) is the same as the ability to produce Yst at temperatures below 30°C. That kind of alkaline environment is present in the end sections of the gastrointestinal tract where *Y. enterocolitica* is usually found. Environmental strains, on the other hand, show the ability to produce an alternative enterotoxin whose mechanism of action is similar to the classical Yst and very comparable to STI. Ramamurthy et al. (1997) called this variant YstB to distinguish it from the original YstA. The *ystB* gene encoding the production of the new variant of Yst was found in 36 out of 304 strains of *Y. enterocolitica* originating from 18 different countries and was always correlated with 1A biotype strains. Additionally, out of 36 strains with the *ystB* gene tested for biological activity using the suckling mouse bioassay, as many as 18 produced the enterotoxin. The role of 1A biotype strains, so far considered non-pathogenic, in the etiology of yersiniosis is also confirmed by the increasing number of clinical cases (Bottone 1999, Grant et al. 1999). One should therefore revise the existing classification into pathogenic and non-pathogenic strains based only on their biotype. Huang et al. (1997) determined the nucleotide sequence of the *ystC* gene, another variant of the classic *yst* gene encoding the enterotoxin production. While *ystA* and *ystB* consist of 30 amino-acids, *ystC* consists of 53 amino-acids and has a larger molecular mass. On the amino-acid level, there is approximately a 50% similarity between C- and N-ending YstC and YstA chains. C-ending 13-amino-acid region of *ystA* and *ystB* correspond to a strongly conservative sequence characteristic for all thermostable toxins (so-called “toxic domain”).

The ability of Yst production has been demonstrated in pathogenic strains isolated from clinical cases of yersiniosis. It indicates that this plays an important role in the etiology of diarrhoea occurring in

the course of the disease. However, not all the strains possessing *yst* genes produce the enterotoxins. It seems to be caused by the *ymoA* gene encoding production of the YmoA protein inhibiting the expression of various genes (Platt-Samoraj et al. 2006). This protein, considered to belong to the growing family of conservative Hha proteins with a small molecular mass, shows similarities to proteins of the H-NS group (Ellison et al. 2006). In intestinal bacteria H-NS proteins play an important role, both as structural proteins and as gene expression modulator, including virulence markers (Dorman 2004). The expression of genes directly responsible for the pathogenicity of *Y. enterocolitica* has been the subject of many studies. It was proved that this process is significantly influenced by temperature, but the mechanism is not entirely clear. It is supposed that YmoA is the key regulator of gene expression by environmental factors, including temperature (Madrid et al. 2007, Banos et al. 2008). It was also proved that the YmoA protein inhibits the expression of the *inv* gene encoding invasion – an important virulence factor responsible for the transport of bacteria cells through M cells (Ellison et al. 2003) and participating in the temperature-dependent production of *Yersinia* outer proteins (Yops) and *Yersinia* adhesin (YadA) – plasmid virulence markers (Cornelis et al. 1991). World literature points to the role of YmoA and related proteins (H-NS) in the regulation of gene expression in various microorganisms, e.g. *E. coli*, whereas there are only few reports on *Y. enterocolitica* (Cornelis et al. 1991, Ellison et al. 2003, 2006, Brzostek et al. 2007, Banos et al. 2008, Fang et al. 2008).

The purpose of our study was to evaluate the distribution of the *ymoA* and *ystA* genes and Yst production by *Y. enterocolitica* isolated from humans and pigs.

Materials and Methods

Bacterial strains

The material for the study consisted of 86 *Y. enterocolitica* strains isolated from humans and 96 *Y. enterocolitica* strains isolated from clinically healthy fattening pigs. The *Y. enterocolitica* strains from humans were isolated in the 2010 and 2011 years from clinical cases of yersiniosis and confirmed by the National Institute of Public Health – National Institute of Hygiene in Poland. Both human and pig strains of *Y. enterocolitica* were then bio- and serotyped according to the PN-EN ISO 10273 standard. *Y. enterocolitica* strains isolated from the fattening pigs mostly belonged to biotype 4 – only 1 strain belonged

to biotype 2. Serotyping gave a positive result in 77 strains with the diagnostic serum for antigen O:3, in 8 with the serum for antigen O:9, whilst 11 strains were found to be non-identified (NI). Most *Y. enterocolitica* strains isolated from the humans belonged to biotype 4, serotype O:3, only 6 strains belonged to biotype 1B, serotype O:8 and 3 belonged to biotype 2, serotype O:9.

DNA isolation

Genomic DNA isolation was performed with the "Genomic Mini" kit (A&A Biotechnology, Gdynia, Poland) designed for the isolation of DNA from bacteria, cell cultures, and solid tissues. The isolation was carried out following the manufacturer's instructions. The purified DNA was stored in a test tubes at -20°C for further analyses.

Primers and multiplex PCR conditions

The multiplex PCR method involved the amplification of four gene fragments: *ystA*, *ystB*, *ystC* and *ymoA*. The primer sequences (synthesised at Oligo, Warsaw, Poland) were obtained from the papers by Platt-Samoraj et al. (*ystA*, *ystB*) (2007), Grant et al. (*ymoA*) (1998) and Bancercz-Kisiel et al. (*ystC*) (2011b).

A multiplex PCR was carried out using HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany) and the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany). The reaction mixture of 20 µl volume contained: about 120 ng of isolated DNA (from 1 to 3 µl), 10 µl HotStarTaq Plus Master Mix 2x, 2 µl CoralLoad Concentrate 10x, 0.1 µl of each of the primers (final concentration 0.5 µM), and the total was supplemented up to 20 µl with RNase-Free Water. The applied reaction conditions were published previously (Bancercz-Kisiel et al. 2011b). Electrophoresis separation was performed in a 2% agarose gel containing 0.5 µg/ml of ethidium bromide, and the size of the obtained products was evaluated by means of a comparison with the standard GeneRuler™ 100 bp Ladder Plus (Fermentas, Lithuania).

Suckling mouse bioassay

The ability to produce enterotoxin by *Y. enterocolitica* isolated from pigs was demonstrated using suckling mouse bioassay (infant BALB/c mice) according to Giannella (1976) with two small modifica-

tions. The first modification was the way by which the supernatant for the inoculation was prepared. In our study each strain was cultured into a tryptic soy broth containing 0.6% yeast extract (TSB-YE) and incubated at 25°C for 48 hours. The second modification was the 2 hours interval between supernatant inoculation and euthanasia, according to Nunes et al. (1981), who showed that the peak of the Yst production takes place between 1 and 2 hours after inoculation. The particular modifications and the reasons for them were precisely described by the authors previously (Bancercz-Kisiel et al. 2011a). Ratio of intestinal mass to the rest of the body mass in the group of three examined sucklings was the basis for the bioassay interpretation. Ratio ≤ 0.074 was regarded as a negative result, 0.075 – 0.082 as a doubtful result and ratio ≥ 0.083 was regarded as a positive result.

The ability to produce Yst by *Y. enterocolitica* isolated from humans was confirmed by the fact that all the strains were isolated from clinical cases of yersiniosis with diarrhea.

Results

Molecular examinations of human and animal *Y. enterocolitica* strains showed that all the studied strains had the *ystA* gene. An amplification of a fragment of the *ymoA* gene was also detected in all the studied strains. No 180 and 134 bp bands indicating, respectively, the presence of the *ystB* and *ystC* gene amplicons were also obtained. The results of the multiplex PCR were documented with photographs of the agarose gels (see example in Fig. 1).

The study of the ability to produce enterotoxin Yst by *Y. enterocolitica* strains isolated from pigs using suckling mouse bioassay showed positive results in 14 cases, constituting 14.58% of all the examined strains. Doubtful results were obtained in case of 33 strains (34.37%), whereas the remaining 49 strains (51.04%) did not show Yst production. The ability to produce Yst by *Y. enterocolitica* isolated from humans were confirmed by the fact that all the strains were isolated from clinical cases of yersiniosis with diarrhea.

The results of multiplex PCR, detecting virulence markers and enterotoxic properties taking into consideration the biotype, serotype and the number of examined *Y. enterocolitica* strain are presented in Table 1.

Discussion

The results obtained in our study reveal several new and interesting data on epidemiology and the pathogenesis of *Y. enterocolitica* infections and corre-

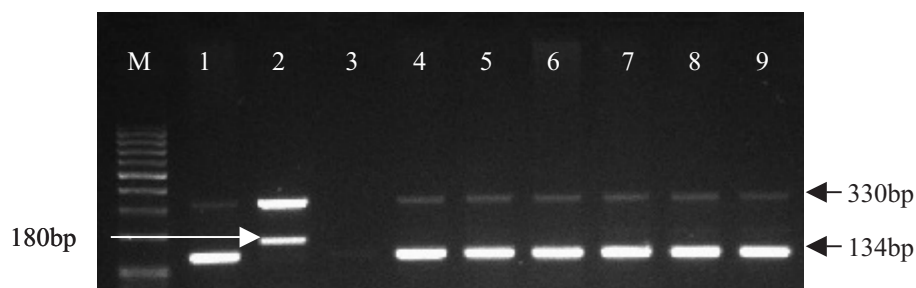


Fig. 1. Electrophoretic separation of products of *ystA*, *ystB*, *ystC* and *ymoA* gene amplification from *Y. enterocolitica* strains in multiplex PCR

M – size marker GeneRuler™ 100bp DNA Ladder Plus (Fermentas).

Lane 1 – positive control, contains DNA isolated from a reference strain of *Y. enterocolitica* O:8 (ACTT 23715).

Lane 2 – positive control, contains DNA isolated from a reference strain of *Y. enterocolitica* O:5.

Lane 3 – negative control, does not contain bacterial DNA, contains *ystA*, *ystB*, *ystC* and *ymoA* gene primers.

Lanes 4 – 6 – products of amplification of *ystA* and *ymoA* gene fragments *Y. enterocolitica* strains isolated from pigs.

Lanes 7 – 9 – products of amplification of *ystA* and *ymoA* gene fragments *Y. enterocolitica* strains isolated from humans.

Table 1. Comparison of the biotypes, serotypes, genotypes and enterotoxic properties of *Y. enterocolitica* strains isolated from humans and pigs.

Source	Biotype	Serotype	Number	Virulence markers				Enterotoxins production		
				<i>ystA</i>	<i>ystB</i>	<i>ystC</i>	<i>ymoA</i>	positive	doubtful	negative
Humans	1B	O:8	6	+	–	–	+	6	–	–
	2	O:9	3	+	–	–	+	3	–	–
	4	O:3	77	+	–	–	+	77	–	–
Pigs	2	NI	1	+	–	–	+	–	–	1
		O:3	77	+	–	–	+	11	26	40
	4	O:9	8	+	–	–	+	1	4	3
		NI	10	+	–	–	+	2	3	5

NI – non-identified

lation between strains isolated from humans and pigs. The most important stage of the presented study was the evaluation of the distribution of the *ymoA* and *ystA* genes and Yst production by *Y. enterocolitica* isolated from humans and pigs. The results of the multiplex PCR developed for detection of four virulence markers in one reaction were compared with the results of an enterotoxicity test (suckling mouse bioassay) of individual *Y. enterocolitica* strains obtained from pigs. Human *Y. enterocolitica* strains with confirmed enterotoxic properties were also molecularly examined. Until now no such studies had been carried out. Grant et al. (1998) searched sequences characteristic for genes encoding enterotoxins production using the hybridisation method; PCR was used only for the *ymoA* gene. Bhagat et al. (2007), on the other hand, used single PCR for *ystA*, *ystB*, *ystC* and *ymoA* genes, while Woźniak-Kosek et al. (2001) only looked for fragments of genes encoding the production of enterotoxins not taking into the account the *ymoA* gene. None of the above mentioned studies included an assessment of the enterotoxicity of *Y. enterocolitica* strains obtained from pigs.

According to the reports of Ramamurthy et al. (1997) based on a study of 304 *Y. enterocolitica* strains, as much as 78.5% of strains categorized as pathogenic had the *ystA* gene, while *ystB* and *ystC* genes in this group were very rare. In the group of non-pathogenic strains, the *ystA* gene was considerably less common (26.1%) than the *ystB* gene (67.4%). The *ystC* gene was detected in only 3 strains of *Y. enterocolitica*: 2 from Japan and 1 from Belgium. In our study all the examined *Y. enterocolitica* strains isolated from pigs showed the presence of *ystA* gene and should be categorized as pathogenic what, in connection with their pathogenic properties, indicates a risk for the health of humans. Classically pathogenic strains isolated from human cases of diarrhea produce thermostable enterotoxin YstA (Delor et al. 1990, Mikulskis et al. 1994, Ramamurthy et al. 1997, Singh and Viridi 2004) and the fragments of the *ystA* gene were detected in all human *Y. enterocolitica* strains examined in our study.

We also proved that the target *ymoA* gene was present in all the studied *Y. enterocolitica* strains, both human and animal. A similar result was obtained in

the study of Grant et al. (1998) concerning 20 biotype 1A strains, where *ymoA* gene amplification products were found in all the studied strains. Also Bhagat et al. (2007) in their molecular tests of biotype 1A strains of *Y. enterocolitica* found sequences characteristic for the *ystB* and *ymoA* genes to be present in all examined strains. There were no reports regarding occurrence of *ymoA* gene together with *ystA* gene. In the context of the above mentioned reports and the authors' own studies we should notice that the presence of *ymoA* or *ystA* genes is insufficient criterion to determine enterotoxic properties of examined *Y. enterocolitica* strain and we should continue our researches. Maybe then we will be able to answer the question why, despite the same biotype, serotype and the presence of *ystA* gene, *Y. enterocolitica* strains show different enterotoxic activity, what was observed in the authors' investigation.

An important finding of the study was also showing that the majority of *Y. enterocolitica* strains, especially isolated from pigs, belonged to biotype 4 which is common in Poland but mainly in humans. Similarly, bioserotypes 4/O:3 and 2/O:9 proved to be predominant, both in humans and pigs. It seems particularly interesting in the context of studies conducted recently by Boghenbor et al. (2006) intended to determine the genotype of *Y. enterocolitica* strains originating from pigs and humans. The study proved that 83-87% of *Y. enterocolitica* strains belonging to serotypes 2/O:9, 3/O:3 and 4/O:3 showed great phylogenetic similarity so not only the typically pathogenic strains included in bioserotype 4/O:3 may cause infection in humans (Boghenbor et al. 2006). The other important finding of our study was showing the absence of biotype 1A strains of *Y. enterocolitica* obtained from pigs. The reliability of this result was confirmed both by serotyping (serotype O:5 was not found) and multiplex PCR (no amplicons of *ystB* and *ystC* genes). This finding is in line with reports of Woźniak-Kosek et al. (2001) but in opposite with reports of other authors showing the predominance of biotype 1A strains in isolates obtained from pigs (Thoerner et al. 2003, Kot et al. 2007).

Bio- and serotyping of *Y. enterocolitica* strains isolated from human cases of yersiniosis showed predominant causative role of bioserotype 4/O:3. Only a few 2/O:9 strains were isolated but significantly more interesting is the fact that 6 most pathogenic 1B/O:8 strains were isolated in Poland. This *Y. enterocolitica* strains considered as prevalent for USA were as yet uncommon in Poland.

Trying to expand the knowledge on mechanisms inducing the production of enterotoxins by demonstrating the correlation between the occurrence of the *ymoA* and *yst* genes and the production of Yst by *Y.*

enterocolitica, we showed that studies should be continued. Using more sophisticated techniques we should define phylogenetic relationship between human and animal *Y. enterocolitica* strains, the possible influence of mutation in *ymoA* and *ystA* genes on the enterotoxin YstA production and the expression level of this genes.

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