Effect of experimental immunization of pigs with a suspension of *Yersinia enterocolitica* selected strains on changes in serum immunoglobulin G levels

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Abstract

The major reservoir of Yersinia enterocolitica and the main source of risk posed to human health are pigs. The objective of this study was to develop a method for pigs immunization with a suspension of immunogenic strains of Y. enterocolitica and to analyze the impact of this immunization on the course of immunological processes in animals, and thus to search for possibilities of preventing or reducing potentially negative effects of Y. enterocolitica experimental infections in pigs.

Key words: *Yersinia enterocolitica, immunization, pigs.*

Introduction

The pathogenicity of *Yersinia spp.* is due to their invasive properties, their capability for proliferation in a host's body and production of toxins [1-4]. A predominating form of yersiniosis are alimentary disorders, although other symptoms are diagnosed in its course as well, including symptoms resembling appendicitis, reactive arthritis, erythema nodosum, micro-abscesses of internal organs, and sepsis [1, 2]. According to data provided in the latest EFSA (European Food Safety Authority) report, yersiniosis is ranked third amongst alimentary zoonoses, after campylobacteriosis and salmonellosis [5]. *Yersinia enterocolitica* is a bacteria widespread in the natural environment, whilst pigs has been acknowledged as its major reservoir and source of infection [1, 6-10]. The effect of infection in pigs includes, mainly, long-term carrier state and excretion of bacteria to the environment, which poses threat to human health. Attempts of creating herds free of *Y. enterocolitica* have so far been unsuccessful owing to a too wide dissemination and a variety of transmission routes of this pathogen [10, 11].

World-wide investigations into experimentally-induced yersiniosis of animals have been conducted for years, however pathogenesis and immunological response in the course of *Y. enterocolitica* infection are still inconclusive. Sparse researches are also available regarding the specific immunoprophylaxis of yersiniosis. This has, to a great extent, been attributed to a long-standing conviction that this bacteria is non-pathogenic to experimental animals, e.g. to rabbits, guinea pigs, rats or mice. A lack of a laboratory experimental model has for years been impairing the development of research addressing this issue. Later studies have demonstrated that some strains of *Y. enterocolitica* are pathogenic to selected breeding lines of laboratory animals, mice in particular [2, 3, 12]. For this reason, most of original papers focused on the specific immunoprophylaxis of yersiniosis are based on experiments conducted on a mouse model. The infection of mouse results in diarrhea, which however is not the only symptom of the disease [2, 3, 13]. The mouse model of *Y. enterocolitica* infections does not always mirror processes ongoing in organisms which

under natural conditions are most frequently infected with this bacteria. For the major reservoir and, thus, the key source of risk posed to human health are pigs, there is a need for extending knowledge on the feasibility of preventing infections with *Y. enterocolitica* in this animal species. Therefore, the objective of this study was to develop a method for pigs immunization with a suspension of immunogenic strains of *Y. enterocolitica* and to analyze the impact of this immunization on the course of immunological processes in animals, and thus to search for possibilities of preventing or reducing potentially negative effects of *Y. enterocolitica* experimental infections in pigs.

Material and methods

Preparation of immunogenic *Yersinia enterocolitica* suspension

The suspension was prepared from *Y. enterocolitica* strains selected out of 60 isolates obtained from internal organs of aborted fetuses, fetal membranes, placentas as well as from vaginal and rectal swabs of sows. Detailed information referring to the origin of samples and bacteriological analyses they were subjected to as well as particular characteristics of isolates applied in the immunogenic suspension were provided in earlier papers [4, 14]. The strains were selected based on immunogenicity assessment using RBA/PKA (respiratory burst activity/potential killing activity) test and MTT (Mitogen Transformation Test). The RBA/PKA test was used to evaluate the effect of *Y. enterocolitica* on the activity of phagocytic cells, whereas the MTT – on the activity of T lymphocytes subjected to the effect of *Y. enterocolitica* cells expressed by the extent of proliferative response to mitogens.

Respiratory burst activity

Metabolic activity of polymorphonuclear (PMN) and morphonuclear (MN) cells was evaluated using the RBA test after cell stimulation with PMA (Phorbol Myristate Acetate, Sigma) described by Secombes [15] in modification by Siwicki *et al.* [16]. Whole blood (100 μl) was poured into 96-well microplates (NUNC), afterwards 100 μl of RPMI 1640 with 0.1% FCS (Foetal Calf Serum, Sigma) were added to each well, and the plates were incubated for 24 h, at a temperature of 4˚C. After incubation, the non-adhered cells were removed by drawing off the fluid. The fluid was next replaced by the addition of 100 μl/well of a 0.1% solution of nitrotetrazolium blue (NBT) in RPMI or a 0.1% NBT solution in RPMI with PMA – at the amount of 1 μl/ml of a 0.1% solution of NBT. In addition, 20 μl of the suspension of the examined strains of *Y. enterocolitica* with density of 10^4 cfu/ml were added to each well. Each sample was analyzed in triplicate. The plates were incubated for 30 min at a temperature of 37˚C. After incubation and medium removal, the cells were washed three times with 70% ethyl alcohol. The production of H_2O_2 by the cells was measured in a microplate reader MRX 1.1 (Dynex, Great Britain) at a wavelength of 620 nm.

Potential killing activity test

Potential killing activity of blood PMN and MN cells was measured with the spectrophotometric method using the PKA test according to Rook *et al.* [17] in modification by Siwicki and Anderson [18]. Whole blood (100 μl) was poured into 96-well plates (NUNC), next 100 μl of RPMI 1640 with 0.1% FCS (Sigma) were added to each plate, and the plates were incubated for 24 h at a temperature of 4˚C. After incubation, the non-adhered cells were removed by drawing off the fluid, which was next replaced by a 0.1% NBT solution in phosphate buffered saline (PBS) containing 18-h culture of *Staphylococcus aureus*. Afterwards, 20 μl doses of a suspension of the examined *Y. enterocolitica* strains with density of 10^4 cfu/ml were added in three replications, and afterwards incubated for 30 min at a temperature of 37˚C. After incubation and medium removal, the cells were washed three times with 70% ethyl alcohol. The plates were dried for 3 min and next 120 μl of 2M KOH and 140 μl of DMSO were added to dissolve formazan. Reading was performed in a microplate reader MRX 1.1 (Dynex) at a wavelength of 620 nm.

Mitogen transformation test

The proliferative response of lymphocytes stimulated with mitogens was performed with the colorimetric method based on MTT tetrazolium salt according to Mosmann [19] in modification by Siwicki *et al.* [16]. Samples of peripheral blood were diluted in a ratio of 1 : 1 with RPMI 1640 medium (Sigma). Lymphocytes were isolated in a Histopaque 1077 gradient (Sigma). The isolated lymphocytes $(1 – 5 \times 10^6)$ were suspended in the RPMI 1640 medium with the addition of 10% FCS (Sigma) and poured into 96-well microplates (NUNC) in doses of 100 μl/well. Afterwards, 100 μl of mitogen were added to each well, i.e. concanavalin A (ConA, Sigma) at the concentration of 64 μg/ml or lipopolysaccharide (LPS, Sigma) obtained from *Escherichia coli* serotype 0111:B4, at the concentration of 160 μg/ml. Next, 20 μl portions of the suspension of the examined *Y. enterocolitica* strains with a density of 10^4 cfu/ml were added to each well, in three replications. The RPMI 1640 medium at the amount of 100 μl/well were used as the control. The plates were incubated for 72 h at a temperature of 37˚C (5% CO_2). After incubation, 10 μl of an MTT (3-[4,5-dimethylazol-2-yl]-5,2diphenyl tetrazolium bromide, Sigma) solution at the concentration of 5 mg/ml PBS were added to each well, and the plates were again incubated for 4 h at a temperature of 37˚C (5% CO_2). Further on, the plates were centrifuged for 15 min at 115 × g, the supernatant was removed and 100 μl of dimethylsulfoxide (DMSO, Polskie Odczynniki Chemiczne SA)

Table 1. Scheme of selection of *Yersinia enterocolitica* strains

Test	I	II	III	IV	kI	kII	kIII	kIV	K1	K2
						Groups				
RBA by PMA		Cells + PMA + analyzed strain			Cells + RPMI medium + analyzed strain				Cells + RPMI medium	Cells + PMA
PKA *Staphylococcus aureus*		Cells + *S. aureus* + analyzed strain			Cells + RPMI medium + analyzed strain				Cells + RPMI medium	Cells + *S. aureus*
Mean ConA		Cells + ConA + analyzed strain			Cells + RPMI medium + analyzed strain				Cells + RPMI medium	Cells + ConA
Mean LPS		Cells + LPS + analyzed strain			Cells + RPMI medium + analyzed strain				Cells + RPMI medium	Cells + LPS

Group I – strains inducing proliferation 10× stronger than the control; Group II – strains inducing proliferation 3-9× stronger than the control; Group III – strains inducing proliferation 1-2× stronger than the control; Group IV – strains inducing proliferation less than 1× stronger than the control; KI-KIV – control with RPMI medium; K1 – control with RPMI not containing Y. enterocolitica; K2 – control with PMA or S. aureus, Con A, LPS, not containing Y. enterocolitica

were added to each well. After 10 min, the absorbance was read in a microplate reader MRX 1.1 (Dynex) at a wavelength of 620 nm.

Selection of strains

In order to classify the isolates to prepare the experimental *Y. enterocolitica* immunogenic suspension, out of the 60 strains examined that had earlier been divided into 4 groups depending on proliferation level, four strains were selected from each group. Thus selected and divided strains were used to prepare mixtures (24-h culture, 3° in McFarland's scale), that were again analyzed with RBA/PKA test and MTT in order to determine the reciprocal effect of the investigated strains on the level of non-specific immunity indices. The scheme of selection of the analyzed strains was presented in Table 1.

Determination of the immunogenic *Yersinia enterocolitica* suspension dose *in vivo*

Animals

In order to determine the optimal dose of the immunogenic suspension, 15 pigs of a hybrid variety PIC were used, with body weight of ca. 20 kg, serologically-negative in examinations for the presence of anti – *Y. enterocolitica* antibodies. The animals were divided at random into three groups and placed in separate rooms, isolated from one another.

All activities involved in the experiment were carried out in accordance with the principles for the care and use of research animals and was approved by the Local Ethic Committee for Animal Experiments (No. 24/N).

Experimental immunization

There were prepared two different doses of a cell suspension of immunogenic *Y. enterocolitica* strains with a density of 2.7×10^9 cfu/cm^3 inactivated with formol. One group of animals was administered subcutaneously 2 ml and the

other 5 ml of the suspension, twice in a 2-week interval. The third group of pigs served as the control and received PBS in the analogous scheme.

The evaluation *in vivo* was conducted after *per os* challenge with a pathogenic *Y. enterocolitica* O:3 strain, at a dose of 10 ml and a density of 2.7×10^9 cfu/cm^3, performed 3 weeks after immunization.

Over the experimental period, the animals were under constant clinical observation (general health status, appetite, internal body temperature, body weight gain) and bacteriological examination.

Antibodies

Serum levels of specific antibodies against Yop (Yersinia outer protein) *Y. enterocolitica* antigen were analyzed with the ELISA test using a commercial kit PIGTYPE® YOPSCREEN (Labor Diagnostic, Leipzig, Germany). The test was conducted following producer's instructions.

Statistical analysis

The statistical analysis of study results was conducted with the Fisher's NIR test (least significant difference) and Tukey's RIR test (reasonable significant difference), using statistical software STATISTICA 6.0.

Results

Selections of strains for preparation of experimental *Yersinia enterocolitica* suspension

The evaluation of *Y. enterocolitica* effect on the activity of phagocytic cells demonstrated significant differences in the impact of the analyzed *Y. enterocolitica* strains on the metabolic and phagocytic activity of PMN and MN cells. Simultaneously, significant differences were noted in the effect of the *Y. enterocolitica* strains examined on the proliferative response of T and B lymphocytes. Based on the

proliferative properties of cells in respect of the control group, the strains examined were divided into four groups. The first included strains having a 10-fold stronger effect on lymphocytes proliferation that the control strains, whereas the second and the third group included strains inducing proliferation respectively 3-9 times and 1-2 times stronger than the control strains. The final fourth group included strains that were inducing proliferation once weaker than the control.

The selected and divided into four groups strains of *Y. enterocolitica* were used to prepare mixtures (24-h culture in 3˚ McFarland's scale), that were again analyzed with the RBA/PKA test and MTT in order to determine the reciprocal interactions of the strains (Tables 2 and 3).

The RBA/PKA test demonstrated a lack of the suppressive effect on phagocytic cells of peripheral blood in all analyzed groups of strains, even in group IV constituting a mixture of *Y. enterocolitica* strains being the weakest in inducing proliferation. All groups of strains exhibited a strong effect on the metabolic and phagocytic activity of PMN and MN cells, yet no significant differences were noted between them.

The MTT demonstrated that groups I and II included strains with the strongest immunogenic properties, very strongly inducing the mitogen-stimulated immunity. In addition, the strains from groups I and II were shown to strongly induce the proliferative response of lymphocytes individually – without the presence of mitogens. For this reason,

those strains were found the most appropriate for preparing the experimental immunogenic *Y. enterocolitica* suspension.

The highly immunogenic *Y. enterocolitica* strains selected for immunogenic suspension preparation constituted the following serobiotypes: O:3/4 – 1 strain, O:5/1A – 4 strains, O:8/1A – 1 strain, and two serologically not typable strains belonging to 1A biotype. Properties of the strains were described in detail in earlier papers [4, 14].

Experimental immunization

Changes in levels of anti-*Y. enterocolitica* antibodies in the course of experimental immunization of pigs, measured with the ELISA, were presented in Fig. 1. Results achieved were expressed as a per cent (%OD) of color reaction inhibition.

The immunization of piglets with the experimental suspension of inactivated highly immunogenic *Y. enterocolitica* strains was inducing the production of anti-Yop *Y. enterocolitica* antibodies in the first week post infection (wpi), when an insignificant increase in OD value was recorded in both immunized groups. Those levels were increasing until 3 wpi, reaching a slightly higher level in group II, where OD was at the level of cutoff values referred to as positive. After the challenge, the immune response in both immunized groups was enhanced and the levels of antibodies reached a multiplied value in respect to the level induced by immunization. The immune response as a result of *per os* infection with *Y. enterocolitica* occurred the earliest and was the

Table 2. Mean optical density of a mixture of selected *Yersinia enterocolitica* strains in the RBA/PKA

| RBA/PKA	Groups of *Y. enterocolitica* strains OD (620 nm)					
	I	**II**	**III**	**IV**	**K1**	**K2**
PMA	0.413	0.505	0.587	0.598	0.487	0.043
Staphylococcus aureus	0.393	0.340	0.437	0.377	0.443	0.048
MTT	Groups of *Y. enterocolitica* strains OD (620 nm)					
	I	**II**	**III**	**IV**	**K1**	**K2**
Mean ConA	1.218	1.306	1.263	1.340	0.729	0.064
Mean LPS	1.359	1.182	1.247	1.115	0.66	0.071

Explanations according to Table 1
OD – optical density

Table 3. Results of selection of *Yersinia enterocolitica* strains used to prepare immunogenic suspension following the scheme presented in Table 1

	Groups of *Y. enterocolitica* strains OD (620 nm)									
	I	**II**	**III**	**IV**	**kI**	**kII**	**kIII**	**kIV**	**K1**	**K2**
Mean ConA	0.428	0.233	0.345	0.326	0.419	0.234	0.469	0.183	0.110	0.144
Mean LPS	0.244	0.254	0.381	0.246	0.493	0.229	0.454	0.225	0.121	0.122

Explanations according to Table 1
OD – optical density
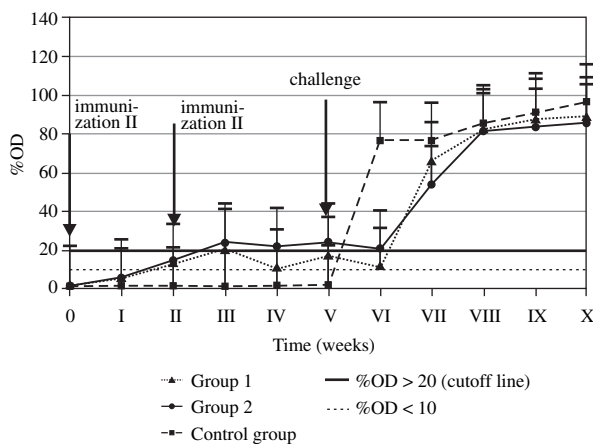
Fig. 1. Levels of IgG anti – Yop for *Yersinia enterocolitica* measured with the ELISA in pigs after experimental immunization and challenge

strongest in the control group. In 3 wpi, the levels of antibodies were alike in all groups and maintained at a similar, high level until the end of the experiment.

No effect of immunization, both the first and the repeated one, was observe on the clinical picture nor body weight gains of the immunized animals. Results of bacteriological analyses and clinical examinations will be described in detail in another paper.

Discussion

Most of investigations addressing immunological processes induced by immunization with *Y. enterocolitica* cells have been conducted with laboratory animals [20-24]. The course of yersiniosis in mice or rabbits differs, to some extent, from its course in humans or in pigs which are the main reservoir of *Y. enterocolitica*. It may, therefore, be speculated that some differences will also be observed upon immunization of laboratory animals.

The pathogenicity of *Y. enterocolitica* is strictly related to the presence of a virulence plasmid (pYV) responsible for many typical properties of a microorganism *in vitro* and *in vivo*, including e.g. calcium dependent growth [2, 13, 25]. In a study by Toyos *et al.* [23], vaccinations were conducted with inactivated and viable bacterial cells of *Y. enterocolitica* O:3, both the Ca^{2+}-dependent and Ca^{2+}-independent ones. This research has demonstrated that the killed bacterial cells, both the Ca^{2+}-dependent and Ca^{2+}-independent ones, assured the same level of immunity when administered intraperitoneally or orally, and that they completely protected mice against oral infection.

In our study, the immunogenic suspension contained *Y. enterocolitica* strains that belong to various biotypes and serotypes, including biotype 1A which is characterized by,

among other things, a lack of virulence plasmids [4, 14]. The main criterion of isolates selection was high immunogenicity demonstrated *in vitro* by means of RBA/PKA test and MTT. No description was found in the available literature regarding earlier investigations with the use of a similar method for the selection of strains to be used for immunization, however based on experimental results of mice immunization it may be presumed that the presence of virulence plasmids is not directly proportional to the immunogenic capability of *Y. enterocolitica* strains, which is also indicated by results of own *in vitro* studies.

In order to elucidate the role of humoral and cellular immunity in the prevention of *Y. enterocolitica* infections Nakajima *et al.* [22] were immunized mice with live and killed cells of the microorganism. Both the live and inactivated vaccines were stimulating the body to produce antibodies, and the titre of agglutinins reached ≥ 1 : 320. Infection through intraperitoneal or intragastric route did not enhance this effect. Alike results were achieved by other research group [25], which also conducted its experiment with the mice model. The applied vaccines, apart from inducing antibodies production, were preventing bacteria excretion with feces once the titre of agglutinins reached ≥ 1 : 320.

In our study, the experimental subcutaneous immunization of pigs with *Y. enterocolitica* cells, likewise in the mice model, induced that production of antibodies. However, the concentration of IgG in pig serum was at a low detection level by ELISA, yet in group II administered the higher dose of the experimental immunogenic suspension, the titres were slightly higher. According to the test's criteria, not in all pigs from group I was the OD recorded at a level above 20, referred to as the cutoff value of the positive reaction.

In our study, the oral infection of the pigs was conducted 3 weeks after the second immunization. In contrast to findings by Nakajima *et al.* [22] reported for mice, the immune response was significantly enhanced and the level of antibodies increased 8 fold within 2 weeks. The increase in the levels of antibodies occurred in all groups, however 1-week retardation of the immune response was observed in the immunized animals, compared to the control pigs. In 4 wpi, levels of antibodies in all groups were at a similar, higher level, being insignificantly higher than in the control group.

The results achieved demonstrated that higher levels of antibodies were obtained in the group of animals administered subcutaneously the higher dose of the experimental immunogenic suspension, i.e. 5 ml 2.7×10^9 cfu/cm^3 twice in a 2-week interval. Immunization conducted in this way will be applied in future investigations aimed at searching for methods of preventing infections or reducing *Y. enterocolitica* shedding in pigs.

Assuming that vaccination has been successful as a means of preventing or reducing the severity of infectious disease [26], investigations conducted with laboratory ani-

mals have proven the feasibility of producing an effective vaccine against yersiniosis. Infections with *Y. enterocolitica* may pose risk especially to public health, hence the practical application of research into the possibility of preventing or reducing infections in livestock is, presumably, only a matter of time, and further investigations in this respect seem to be substantiated.

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