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Prevalence, biofilm formation and virulence markers of *Salmonella* sp. and *Yersinia enterocolitica* in food of animal origin in Poland

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ABSTRACT

Examination of 330 food samples (meat, white raw sausage, smoked meat and cheeses) was conducted and the *Salmonella* spp. and *Y. enterocolitica* prevalence were determined. For isolated strains of *Salmonella* spp., it was determined whether they were of *S.* Typhi, *S.* Typhimurium or *S.* Enteritidis serotypes. Isolated *Y. enterocolitica* strains were biotyped. Moreover, the strains' ability to form a biofilm and presence of virulence factors was determined. *Salmonella* spp. were found in 16 (5.5%) samples, whereas *Yersinia enterocolitica* in 7 (2.1%) samples. Serotyping resulted in classifying 4 strains as *S.* Typhimurium, 2 as *S.* Enteritidis and none as *S.* Typhi. Ten strains were not classified as any of the determined serotypes. The gene *inv*A was found in all *Salmonella* strains and *spv*C was found in 3 (18.7%) strains. All of the *Y. enterocolitica* were classified as biotype 1A, none of the strains had the genes *ail* or *yst*A, and the *yst*B gene was found in five strains. None of the 16 strains of *Salmonella* spp. and the 7 strains of *Y. enterocolitica* showed any strong ability to form a biofilm, while 7 strains of *Salmonella* spp. and 3 strains of *Y. enterocolitica* showed a moderate ability to form a biofilm.

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1. Introduction

Salmonella spp. and *Yersinia entrocolitica* are gram-negative, intestinal pathogenic bacteria of the Enterobacteriaceae family. According to annual reports of the European Food Safety Authority (EFSA, 2015), *Salmonella* spp. and *Yersinia enterocolitica* are the second and third most common enteropathogens responsible for food poisoning, respectively. Meat is regarded as the main source of these infections, with *Y. enterocolitica* being isolated mainly from pork, whereas *Salmonella* spp. is isolated from poultry (Atobla, Karou, Dadie, Niamke, & Dje, 2012; Baumgartner, Kőffer, Suter, Jemmi, & Rohner, 2008; Graziani et al., 2013; Proroga et al., 2016; Simonova, Borilova, & Steinhauserova, 2008). Apart from the main reservoirs, both of them cause food poisonings after the consumption of nearly every type of food, including dairy products, fish, vegetables and fruit (Chajęcka-Wierzchowska, Zadernowska, Kłębukowska, & Łaniewska-Trokenheim, 2012; Grahek-Ogden, Schimmer, Cudjoe, Nygard, & Kapperud, 2007; Wang et al., 2016; Zadernowska, Chajęcka-Wierzchowska, & Kłębukowska, 2014) Although both bacteria originate in the intestines and are in one family, they differ in terms of growth conditions and methods of isolation. While all of the 2500 *Salmonella* serovars are regarded as pathogenic, only three *Yersinia* species: *Y. pestis* (currently of marginal importance), *Y. pseudotuberculosis* and *Y. enterocolitica* are considered to be pathogenic (Fàbrega & Vila, 2012). Of the latter, there are six biotypes which are highly diverse in terms of their pathogenicity. The most pathogenic biotypes include biotype 1B and serotype O:8, which for many years has been called the "American serotype" because of its area of occurrence. However, probably due to the huge export and import of products and nearly unlimited transfer of people during the past years, it has spread to European countries. Since 2004, a gradual increase in the number of strains with this serotype has been observed in Poland. According to the literature, *Y. enterocolitica* with the bio-serotype of 1B/O:8 is currently the second most common bio-serotype isolated from clinical material in Poland. However, there is not much data on the occurrence and biotype diversity of *Y. enterocolitica* in food (Zadernowska, Chajęcka-Wierzchowska & Łaniewska-Trokenheim, 2014).

The aim of the study was to analyse the incidence of *Salmonella* spp. and *Y. enterocolitica* in raw meat and food products of animal origin, to identify *Salmonella* spp. as serotypes *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis and *Y. enterocolitica* as a biotype. Moreover, the aim of the study was to determine the ability to form

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biofilm by the isolated pathogens and to determine the incidence of selected factors of virulence of the bacteria.

2. Materials and methods

2.1. Sampling

A total of 330 samples were obtained from several local markets in Olsztyn, Poland. Samples were consisted in poultry raw meat (duck, chicken and turkey; n = 64), beef meat (n = 58), pork meat (n = 60), minced pork and beef meat (n = 36), minced poultry meat (n = 36), cured meats (sausages, smoked meats, offal products; n = 54), fresh sausage ("white raw sausage"; n = 33), cheese (hard, soft, blue, and surface ripened; n = 22). All the samples were kept at 4 °C during transport and during storage before analysis. The analysis was done within 4 h after purchase.

2.2. Bacterial isolation and identification

2.2.1. Yersinia enterocolitica

For *Yersinia* isolation, 25 g of each sample was added to 225 ml of ITC – irgasan, ticarcillin, and potassium chlorate medium (according to PN-EN ISO 10273:2003), incubation at 25 °C for 48 h). Next, a loopful was streaked onto a CIN agar plate (cefsulodin, irgasan, and novobiocin) (Merck Millipore) and incubated at 30 °C for 48 h. Further biochemical identification of 1–5 typical colonies from each CIN plate was carried out according to the PN-EN ISO 10273:2005 standard, to make a preliminary selection of potentially pathogenic *Y. enterocolitica* strains. Presumptive *Yersinia* colonies were identified by biochemical tests using the API 20E (bioMérieux 20100). Biotyping of *Y. enterocolitica* were then done by biochemical assays (Table 1).

2.2.2. Salmonella sp.

For *Salmonella* sp., 25 g of each sample was added to 225 ml of buffered peptone water and homogenized using a stomacher, next incubated at 37 °C for 16–20 h. One ml was transferred to 10 ml selenite cystine broth and incubated for 20–24 h at 37 °C. Plating carried on XLD agar (Merck Millipore) and incubated at 37 °C for 24 h. The plates examined for typical colonies of salmonella (red with black center). Presumptive *Salmonella* colonies were confirmed by biochemical test (Indole, Methyl Red, Voges-Proskauer, Citrate and urease and glucose (TSI). (according to PN-EN ISO 6579:2003).

2.3. DNA isolation

Genomic DNA isolation was performed with the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions and was stored at −20 °C for further analyses.

Table 1
Biochemical tests used for biotyping *Yersinia enterocolitica*.

Biochemical tests	Biotype					
	1A	1B	2	3	4	5
Tween-esterase	+	+	−	−	−	−
Esculin	+	−	−	−	−	−
Piraziminidase	+	−	−	−	−	−
Indol	+	+	(+)[a]	−	−	−
Xylose	+	+	+	+	−	D[b]
Trehalose	+	+	+	+	+	−

[a] Weak.
[b] Delayed.

2.4. Presence of ail, ystA and ystB genes in Yersinia enterocolitica

Triplex PCR was carried out to amplify three *Y. enterocolitica* genes: *ail, ystA* and *ystB*. The applied primer sequences and temperature profiles were described by Bancerz-Kisiel, Szczerba-Turek, Lipczyńska, Stenzel, and Szweda, (2012) (Table 2).

2.5. Presence of invA and spvC genes in Salmonella sp.

The *Salmonella* isolates were analyzed by PCR to detect the presence of two virulence genes: *inv*A and *spv*C. PCR was performed with primers and conditions described previously (Table 2).

2.6. Salmonella serotyping

Serotyping of *Salmonella* strains was performed using a Multiplex PCR with primers and conditions described previously (de Freitas et al., 2010), specific for the *Salmonella* sp., *S.* Enteritidis, *S.* Typhi and *S.* Typhimurium (Table 2).

2.7. Electrophoresis condition

The size of the products was evaluated by means of a comparison with the standard mass of GeneRuler 100-bp Ladder Plus (Thermo Scientific, Fermentas, Poland). PCR reaction mixture were analyzed by electrophoresis through a 1.5% agarose gel (Promega, Poland) in 1× TBE buffer pH 8.3. Gels were stained with ethidium bromide (Sigma-Aldrich) and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene) and analyzed using the program Gene Tools (Syngene).

2.8. Detection of biofilm formation by the microplate (MP) method

The method for assessment of biofilm formation by the MP method was based on the techniques proposed by Stepanović, Ćirković, Ranin, and Svabić-Vlahović (2004) with some modifications described previously (Chajęcka-Wierzchowska, Zadernowska, & Łaniewska-Trokenheim, 2016). Wells of a sterile 96-well flat-bottomed sterile polystyrene microtiter plates (Nunc) were filled with 200 μL of fresh sterile broth BHI (Merck Millipore). 20 μL of overnight cultures of each strain with a cell density of 1×10^9 cells/ml were added in triplicate, onto a 96-well. Negative control wells contained broth only. The plates were covered and incubated aerobically at 36 °C for 24 h. The bacterial suspension was aspirated and each well was washed three times with 250 μL of PBS buffer (Sigma). After that, the biofilm was fixed with 200 μL of ethanol (99%) for 15 min, and was later removed. The plates were dried at room temperature, stained with a 200 μL of crystal violet solution used for Gram staining (Merck Millipore) for 5 min, washed in running water until unbound crystal violet was removed and dried at room temperature. The dye bound to the adherent cells was resolubilized with 160 μL of 33% (v/v) glacial acetic acid (Sigma) per well.

Absorbance was read using an Infinite M1000 PRO plate reader (Tecan) at 570 nm. The optical density (ODs) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the cut-off OD (OD_c) which was defined as three standard deviations above the mean OD of the negative control. The following classification was used for the determination of biofilm formation: no biofilm production ($OD \leq ODc$), weak biofilm production ($ODc < OD \leq 2\times ODc$), moderate biofilm production ($2\times ODc < OD \leq 4\times ODc$) and strong biofilm production ($4\times ODc < OD$).

Table 2
Primers used for the amplification of the *Salmonella* serotypes and *Salmonella* and *Yersinia enterocolitica* virulence genes.

Bacteria/target gene	Primers	Sequence 5′–3′	Size (bp)	References
Salmonella sp.	OMPCF	ATCGCTGACTTATGCAATCG	204	de Freitas et al., 2010
	OMPCR	CGGGTTGCGTTATAGGTCTG		
S. Enteritidis	ENTF	TGTGTTTTATCTGATGCAAGAGG	304	
	ENTR	TGA ACT ACG TTC GTT CTTCTG G		
S. typhi	ViaBF	CAC GCA CCA TCA TTT CAC CG	738	
	ViaBR	AAC AGG CTG TAG CGA TTT AGG		
S. Typhimurium	TyphF	TTG TTC ACT TTT TAC CCC TGA A	401	
	TyphR	CCC TGA CAG CCG TTA GAT ATT		
Salmonella sp. virulence genes				
*inv*A	F	ACAGTGCTCGTTTACGACCTGAAT	244	Bhatta et al., 2007
	R	AGACGACTGGTACTGATCGATAAT		
*spv*C	F	ACTCCTTGCACAACCAAATGCGGA	571	
	R	TCTCTTCTGCATTTCGCCACCATCA		
Yersinia enterocolitica virulence genes				
ail	F	TAGTTCTCTAATAGCCTGTTTATC	351	Bancerz-Kisiel et al., 2014; Platt-Samoraj et al., 2006
	R	ACTATCTGAGATGATTAGAATCG		
*yst*A	F	GTCTTCATTTGGAGGATTCGGC	134	
	R	AATCACTACTGACTTCGGCTGG		
*yst*B	F	TGTCAGCATTTATTCTCAACT	180	
	R	GCCGATAATGTATCATCAAG		

3. Results

The analysis of 330 samples of materials and products of animal origin showed 16 (5.5%) positive results for *Salmonella* spp. and 7 (2.1%) for *Yersinia enterocolitica*. *Salmonella* spp. were found in poultry raw meat – 6 (9.4%), in beef meat – 2 (3.4%), pork meat – 2 (3.3%), minced pork and beef meat – 1 (2.8%) and in fresh sausage – 2 (6.1%). *Yersinia enterocolitica* were found in poultry raw meat – 2 (3.1%), pork meat – 3 (5%) and minced pork and beef meat – 2 (5.5%) (Table 3). No sample was found to contain both bacteria. As a result of serotyping, 4 strains were classified as *S.* Typhimurium, 2 as *S.* Enteritidis and none of the isolated strains was classified as *S.* Typhi. Ten strains were not classified as any of the serotypes under study. The gene *inv*A, which encodes a protein InvA playing an important role in the virulence processes, e.g. in penetration of the epithelial cells of the intestines by *Salmonella* spp., was found in all of the *Salmonella* spp. On the other hand, *spv*C, also responsible for virulence, was found in only 3 strains. None of the 16 strains of *Salmonella* spp. demonstrated a strong ability to form biofilms, 7 strains demonstrated a moderate ability to form it, and a low ability was demonstrated by 7, whereas 2 strains were found to be unable to form a biofilm (Table 4). All of the strains of *Y. enterocolitica* were classified as biotype 1A, none of them had the gene *yst*A responsible for the production of enterotoxin YstA or the gene *ai*l responsible for the production of adhesin Ail. The gene *yst*B, responsible for the production of enterotoxin Ystb, was found in 5 out of 7 strains. Examination of the biofilm production process has shown that none of the strains showed a strong ability to produce it, 3 strains showed a moderate ability, one had poor ability, and 3 strains showed no ability to produce biofilm (Table 5).

Table 3
Occurrence of *Salmonella* and *Yersinia enterocolitica* in food of animal origin.

Samples	Number of samples	Number of positive samples (%)	
		Salmonella	*Yersinia enterocolitica*
Poultry raw meat (duck, chicken, turkey)	64	6 (9,4)	2 (3,1)
Beef meat	58	2 (3,4)	0
Pork meat	60	2 (3,3)	3 (5,0)
Minced pork and beef meat	36	1 (2,8)	2 (5,5)
Minced poultry meat	36	3 (8,3)	0
Cured meats (sausages, smoked meats, offal products)	54	0	0
Fresh sausage (“white raw sausage”)	33	2 (6,1)	0
Cheese (hard, soft, blue and surface ripened)	22	0	0
Total	330	16 (5,5)	7 (2,1)

4. Discussion

Salmonella spp. occurred in 5.5% of the tested samples which proves the high level of microbiological safety of materials and products of animal origin commercially available in Poland. The highest percentage of positive samples (9.4%) was found in poultry raw meat. A study conducted by Dallal et al. (2010) in Tehran (Iran) revealed the presence of the bacteria in 33% (379) of the meat samples, with *Salmonella* spp. found in 45% of poultry raw meat samples. A study conducted by Domınguez, Gomez, and Zumalacarregui (2002) revealed the presence of *Salmonella* spp. in 35.83% (198) of poultry raw meat samples bought at retail outlets in Spain. *Salmonella* spp. is not a homogenous group of microorganisms; they make up two species and over 2500 various serotypes, all of them pathogenic to humans. Usually, routine tests do not determine the bacteria serotype and it is only identified as *Salmonella* spp. In regard to poultry raw meat, the European Commission amended regulations No 2160/3003 and 2073/2005 in 2011. These regulations placed a strong emphasis on detection and identification of serovars *S.* Enteritidis and *S.* Typhimurium. This was mainly due to the fact that according to the data provided by the European Food Safety Authority, 80% of salmonellosis were caused by serotypes *Salmonella* Enteritidis and *Salmonella* Typhimurium. In this study, we classified 6 (37.5%) out of the 16 strains as the two serotypes, which – considering the number of serotypes – is a high percentage. Nair et al. (2015) examined 13 strains of *Salmonella* spp. isolated from food from various parts of India, of which 10 were classified as serovar *S.* Enteritidis and 1 as serovar *S.* Typhimurium. As in this study, no strain was classified as serovar *S.* Typhi. These authors also examined 9 environmental strains, 6 of which were classified as *S.* Typhi and 1 as *S.* Typhimurium.

All of the *Salmonella* spp. identified by biochemical methods had the *inv*A gene. It is located on a chromosome and is typical of the genus and is responsible for transfer to epithelial cells.

Table 4
Serotypes, virulence factors and biofilm forming ability in *Salmonella* strains isolated from meat samples.

No.	Strain	Source	*Salmonella* spp. serotype	Virulence genes		Biofilm formation
				*inv*A	*spv*C	
1	4s	fresh sausage	*S.* Typhimurium	+	+	weak
2	16s	fresh sausage	*S.* Typhimurium	+	−	weak
3	14s	poultry meat	*S.* Typhimurium	+	−	weak
4	19s	minced poultry meat	*S.* Enteritidis	+	+	weak
5	30s	minced pork and beef meat	*Salmonella* sp.	+	−	moderate
6	26s	pork meat	*Salmonella* sp.	+	−	moderate
7	29s	minced poultry meat	*Salmonella* sp.	+	−	moderate
8	17s	pork meat	*Salmonella* sp.	+	−	no biofilm producer
9	15s	beef meat	*Salmonella* sp.	+	−	moderate
10	5s	poultry meat	*Salmonella* sp.	+	−	moderate
11	10s	minced poultry meat	*Salmonella* sp.	+	−	weak
12	9s	beef meat	*Salmonella* sp.	+	−	moderate
13	23s	poultry meat	*Salmonella* sp.	+	−	moderate
14	24s	poultry meat	*S.* Enteritidis	+	+	weak
15	6s	poultry meat	*Salmonella* sp.	+	−	weak
16	25s	poultry meat	*S.* Typhimurium	+	−	no biofilm producer

Table 5
Biotypes, virulence factors and biofilm forming ability in *Y. enterocolitica* strains isolated from meat samples.

No.	Strain	Source	Biotype	Virulence gene			Biofilm formation
				ail	*yst*A	*yst*B	
1	*Y. enterocolitica* R1	pork meat	1A	−	−	+	moderate
2	*Y. enterocolitica* O11	pork meat	1A	−	−	+	moderate
3	*Y. enterocolitica* H7	pork meat	1A	−	−	+	no biofilm producer
4	*Y. enterocolitica* R6	poultry meat	1A	−	−	−	no biofilm producer
5	*Y. enterocolitica* R9	poultry meat	1A	−	−	+	moderate
6	*Y. enterocolitica* O12	minced meat	1A	−	−	+	no biofilm producer
7	*Y. enterocolitica* K4	minced meat	1A	−	−	−	weak

Furthermore, the gene *spv*C, encoded on plasmids and responsible for survival in the host's cells, was found in 2 strains of *S.* Enteritidis and in one strain of *S.* Typhimurium (Kumar, Balakrishna, & Batra, 2006). Considering the fact that it is encoded on plasmids, *Salmonella* may have acquired it by horizontal transfer.

The pathogens' ability to form biofilms has a twofold meaning. From a medical point of view, the ability to form it is a feature which proves the strain's virulence. Components of a biofilm matrix protect bacteria against an immune attack (e.g. phagocytosis) and against penetration of chemiotherapeutics (antibiotics). However, in terms of their industrial significance, strains which can form biofilms of manufacturing surfaces can pose a big problem; they are more difficult to remove from those surfaces, they can be less sensitive to disinfectants and they often cause secondary (cross) infections in the food industry (Shi & Zhu, 2009). The ability to form biofilm is largely a strain-related feature. None of the strains of the *Salmonella* and *Y. enterocolitica* species showed a strong ability to produce biofilms. Nair et al. (2015) examined 40 strains of *Salmonella* spp. isolated from food, environmental and clinical samples. None of the strains examined by these authors had a strong ability to form biofilms. On the other hand, a study by Isoken (2015) showed 15 out of 32 *Salmonella* strains to be able to form a strong biofilm. The majority of the *Y. enerocolitica* strains were characterised by a no biofilm producer, or weak ability to form a biofilm, which has been confirmed by reports of other authors. Kot, Piechota, Zdunek, Borkowska, and Binek (2011) showed a high percentage of *Y. enterocolitica* strains of the biotype 1A (38.1%) to have adhesive properties.

Neither *Salmonella* spp. nor *Yersinia enterocolitica* were isolated from any of the cured meats and cheeses samples examined under study (Table 3). They are not thermoresistant and they die at the temperature of pasteurisation; however, there have been many reports which indicate their presence in cold meat and in cheese. Cross-contamination and recontamination is regarded as the main reason for the presence of the bacteria in ready-to-eat food, which was given thermal treatment in the production process. Carrasco, Morales-Rueda, and García-Gimeno (2012) quoted World Health Organization (WHO) data and reported that 25% of all food poisonings are caused by cross-contaminations resulting from a failure to observe good production and hygiene practices.

The *Yersinia enterocolitica* (n = 7; 2.1%) isolated from the samples were classified as biotype 1A. Despite data from clinical trials conducted by Rastawicki, Szych, Gierczynski, and Rokosz (2009) which indicate a considerable increase in the number of isolated strains in biotype 1B, our study has not revealed such a tendency among the strains isolated from food. Biotype 1A is isolated very frequently from environmental and food samples and has not been regarded as pathogenic for many years. However, there has been an increasing number of reports suggesting that strains of this biotype can be pathogenic to humans (Söderqvist, Boqvist, Wauters, Vågsholm, & Thisted-Lambertz, 2012). The isolated strains were not found to contain the two main genes *ail* and *yst*A which encode chromosomal virulence markers – proteins Ail and YstA, which are regarded as the main determinants of pathogenicity of strains in this species (Bancerz-Kisiel, Szczerba-Turek, Platt-Samoraj, Socha, & Szweda, 2014; Platt-Samoraj et al., 2006). Adhesin Ail participates in adhesion and invasion *in vivo* and protects *Y. enterocolitica* against the bactericidal action of complement proteins. Enterotoxin YstA activates guanylyl cyclase in intestinal villi, which results in an increase in cGMP concentration, leading to a consequent loss of ability to absorb liquids and to accumulate liquids in the intestine (Revell & Miller, 2001). On the other hand, the presence of gene *ystB* was detected in five out of seven strains. The nucleotide sequence of the *yst*B is 73.5% homologous with the nucleotide sequence of

the *yst*A gene. It has been confirmed by reports from other authors that this gene is often found in strains of biotype 1A. Although strains of biotype 1A (generally regarded as non-pathogenic) are usually free of virulence plasmids and other known chromosomally encoded factors of virulence, the presence of gene *yst*B has been detected in at least several strains of biotype 1A which are pathogenic to humans (Tennant, Grant, & Robins-Browne, 2003). Kot et al. (2011) and Platt-Samoraj et al. (2006) found the gene *yst*B in all of the strains isolated from pigs. Thoerner et al., found the gene *yst*B in 89% of strains of biotype 1A isolated from patients with diarrhoea, in 87.3% of those isolated from healthy and infected animals and in 80% from food of plant origin and from environmental samples (Thoerner et al., 2003).

5. Conclusions

This study has shown a high safety level for meat and its products available on the Polish market. However, as food infections are still present (which mainly applies to poultry meat), food inspection, diagnostics and precise identification of infections are very important. It is also necessary to raise consumer awareness regarding secondary infections, which can occur during the transport of meat from a shop or during its processing.

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