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OCCURENCE OF YERSINIA ENTEROCOLITICA IN SLAUGHTER PIG TONSILS

Milena Alicja Stachelska

Institute of Food Technology and Food Service
Lomza State University of Applied Sciences, Lomza, Poland

E-mail: mstachelska@pwsip.edu.pl

Abstract: The detection and isolation of food-borne pathogenic Yersinia enterocolitica strains of animal origin are not easy tasks. Over the few last years there have been a lot of classical microbiological and immunochemical methods successfully complemented in combination with the molecular based techniques. In the present study, an optimized protocol for Real-time PCR and a proper system for detection of inv gene in Y. enterocolitica are the subjects of investigation. The selected primer pair MP1/MP2 with the MPFR as a positive control and ®TaqMan probe were found to be successful in detection of pathogenic Y. enterocolitica strains in pig tonsils. 52 out of 139 samples (37.4%) were positive for the inv gene. As the conventional culture method is not a reference method, the PCR products amplified from pig tonsil samples were positively verified by using Real-time PCR, which is a rapid and specific Real-time PCR method for the detection of pathogenic Y. enterocolitica in food. It seems to be a superior alternative for the actually present detection methods which provide huge possibilities for the identification of foods at risk for Y. enterocolitica contamination. The protocol might be efficient in detection of pathogenic Y. enterocolitica strains in clinical samples like meat, milk and faeces.

Key words: inv gene, optimization, Real-time PCR, Yersinia enterocolitica, pig tonsils.

Introduction

Yersinia enterocolitica belongs to foodborne pathogens and is responsible for provoking different symptoms of illness in humans. They include mild diarrhoea, immunological problems and potentially lethal septicaemia. One of the most frequent problems is acute gastroenteritis appearing usually inyoung children. Y. enterocolitica is commonly subdivided into bioserotypes according to the variety of biochemical and serological tests as well as molecular technique including Real-time PCR. Human pathogenic Y. enterocolitica involve biotypes 1B, 2, 3, 4 and 5, whereas strains belonging to biotype 1A are usually considered to be non-pathogenic, and they are isolated from the environment. The bioserotypes responsible for causing human diseases in Poland include bioserotype 4/O:3 and on a smaller scale bioserotype 2/O:9 [1]. The main source of foodborne Y. enterocolitica are tonsils of slaughter pigs. Pigs are considered to be the only food animals which are a reservoir of these pathogenic bioserotypes. There have been many studies carried out which proved that tonsils are regarded to be the main source for the contamination of pig offal and carcasses.

It is known that pathogenic Y. enterocolitica can be transmitted from tonsils to pig organs at slaughter, and it might lead to carcass contamination. As a result, it may constitute a risk of the foodborne transmission to humans. Furthermore, there is a strong need to examine the microbiological risk assessment of Y. enterocolitica in slaughter pigs. In order to isolate pathogenic Y. enterocolitica, an International Standard Organization method is recommended for food samples as well as for pig tonsils [2]. It is not easy to isolate pathogenic Y. enterocolitica from naturally contaminated samples because the standard culture methods for the recovery of Y. enterocolitica take time-consuming enrichment steps followed by plating on selective media [3].

Pathogenic strains of the Y. Enterocolitica have a huge influence on the epidemiological increase in diseases related with consumption of contaminated foods leading to social and healthcare problems. In spite of the evident impact on the food of animal origin in the transmission of yersiniae, it occurs that the isolation of pathogenic strains is often a very laborious and inefficient task in the proper detection of Y. Enterocolitica in pork meat [4]. Pigs are mainly considered to be a reservoir and factor in the transmission of pathogenic strains. The pathogenic potential of Y. enterocolitica is characterised by complex interaction of chromosomal and plasmid genes. The presence of a70 kb plasmid (pYV, plasmid Yersinia virulence) and specific chromosomal determinants (genes inv, ail, yst) are essential in the full expression of pathogenic potential [5].

This pathogen is psychrophilic, which means that it is able to grow and multiply in low temperature during the storage of food products. This is the reason why chilled and frozen foods might be potentialreservoirs and sources of infection as well as the factors responsible for transmission of pathogenic yersiniae strains and sources of food-borne diseases. During the last few years classical microbiological

and immunochemical methods for detection of food-borne pathogens have been successfully developed in combination with DNA-based methods [6]. Apart from the direct hybridization techniques, most of these methods involve in vitro amplification. Real-time PCR is the most successful method in the detection of Y. enterocolitica up to now, and it is based on in vitro amplification of bacterial DNA. It possesses a huge potential for fast and selective detection of microorganisms [7]. Its principle relies on the detection of a fluorescent signal that is proportional to an amount of DNA belonging to food-borne strains [8].

The aim of the present study was to assess the appearance of enteropathogenic Yersinia in slaughterhouses in Poland using the isolation methods and Real-time PCR method for confirmation of pathogenicity of Y. enterocolitica taken from pig tonsils in Poland. Furthermore, the isolation methods and direct plating in order to isolate pathogenic Y. Enterocolitica from pig tonsils were the subject of investigation [9]. The study involved the optimization of a Real-time PCR protocol based on the inv gene in order to detect Y. enterocolitica. This will enable the detection of pathogenic yersiniae in a variety of different samples like meat, milk, faeces.

Materials and Methods: Sampling

Pig tonsils coming from 139 pigs which were slaughtered in one abattoir in Poland were collected during 15 sampling visits. The animals were delivered from 32 different slaughter-batches, and 5 animals were randomly sampled per batch. Tonsils were taken aseptically immediately after evisceration, placed into sterile plastic bags and delivered to the laboratory under chilled conditions where they were subjected to further examination within 5 h after collection.

Materials and methods: Isolation

The samples of tonsils were examined for the presence of pathogenic Y. enterocolitica by direct plating and different enrichment protocols according to the ISO method. Tonsil samples were aseptically cut into small pieces, and 10g of tonsils were put into sterile stomacher bag. The samples were homogenized with 90ml of 0,1% peptone water (Oxoid, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). 0,1ml of such homogenate was plated on CIN agar plates by a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). CIN agar plates are cefsulodin-irgasan-novobiocin agar plates (Yersinia Selective Agar Base and Yersinia Selective Supplement, Oxoid, UK). CIN agar plates were incubated at 30°C for 24h and investigated for characteristic Yersinia colonies using a stereo microscope with Henry illumination (Olympus). Suspected colonies were taken by a sterile loopful and incubated in trypton soy broth (PCA, Oxoid, UK) at 30°C for 24h.

Materials and Methods: Purification of DNA from Food-borne Pathogens Using DNA Bacteria Kit

1ml of enrichment culture was pipetted into a 2ml microcentrifuge screw-cap tube and was centrifuged at 13 000 × g for 5 min. Then the supernatant was discarded using a pipet. Care was taken not to disrupt the pellet. Then 200μl of Fast Lysis Buffer was added to the bacterial pellet, the tube was tightly capped and the pellet was resuspended by vigorous vortexing. Then the microcentrifuge tube was placed into a thermal shaker (800rpm) set to 100°C. The sample was heated for 10 min. The sample was removed and cooled to room temperature (15-25°C) for 2 min. The tube was centrifuged at 13 000 × g for 5 min. Then 100μl of the supernatant was transferred to a fresh 1,5ml microcentrifuge tube.

Materials and Methods: Preparing the PCR Assay

Such supernatant was the subject of further investigation on Real-time PCR (Rotor Mx3000P Stratagene Agilent Technologies, USA) using PowerChek^{TM} Yersinia enterocolitica Real-time PCR Kit Protocol. The kit was used to detect the specific sequence of inv gene for Y. enterocolitica. This kit contained Real-time PCR MasterMix with enzyme components and the specific primer/probe set for the test by Real-time assay and for the Internal Control (IC) system for the reliability of results. This is a qualitative Duplex Real-time PCR test which includes the amplification of pathogen specific gene (inv) and the Internal Control (IC) with specific primers and probes labelled with the fluorescent dyes. The target sequences are detected by the FAM and VIC (HEX) channels. During PCR amplification

Table 1: PCR Reaction Mixture

Composition	**Volume**
Primer/Probe Mix	4μl
2 × Real-time PCR Master Mix	10μl
Template DNA (Control DNA or DNA isolated from food-borne pathogens)	5μl
TE buffer (PCR water, negative control)	1μl
Total	**20**μl

the forward and reverse primers hybridize to the pathogen DNA. A fluorogenic probe is included in the same reaction mixture which consists of an oligonucleotide labelled with a 5'-reporter dye and a downstream 3'-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in

fluorescence is detected on a range of Real-time PCR platforms. The total reaction volume is $20\mu l$, the volume of DNA is $5\mu l$. The reaction mixture was prepared according to the Table 1.

Materials and methods: The Thermal Profiles

Table 2: PCR Conditions

Temperature	Time	Cycle
95°C	10 min	1
95°C	15 sec	40
60°C *	1 min	

* Detection of the fluorescence at this step

Materials and Methods: Data Analysis

The fluorescent curves were analysed on FAM and VIC (HEX) fluorescence detection channels (Table 3). The presence or absence of Y. enterocolitica specific gene was assessed by analysing the Real-time PCR result.

Table 3: Specific Detection on Fluorescence Channels

Target Gene	Fluorophore
inv	FAM
IC	VIC (HEX)

The signal is considered to be positive if the corresponding fluorescence accumulation curve crosses the threshold line. Results are accepted as relevant if both positive and negative controls of amplification are passed. Amplification of DNA Y. enteroclitica is analysed in the FAM fluorescence channel. The specific amplification of Internal Control is analysed in the VIC (HEX) channel.

Table 4: Results Analysis

Yersinia spp. FAM Channel	Internal Control VIC/HEX Channel	Results interpretation
positive	positive	positive
negative	positive	negative
positive	negative	positive
negative	negative	incorrect

Results and Discussion

Pathogenic Y. enterocolitica were isolated from 52 out of 139 tested tonsil samples (37.4%). Table 5 presents the randomly taken samples of pig tonsils which occurred to contain food-borne pathogenic Y. enterocolitica strains. Threshold (dR) for HEX channel was 1617.474 and for FAM channel was 466.313.

Table 5: Yersinia enterocoliticaSstrains Uused for the Sselectivity Ttest of the Aassay

Isolate number	Source	FAM Ct-value	VIC Ct-value	Results interpretation
E1 (positive control)	Control DNA	20.96	30.16	positive
E2	Pig tonsils	21.29	30.23	positive
E3	Pig tonsils	32.76	27.80	positive
E4	Pig tonsils	37.63	26.98	positive
E5	Pig tonsils	35.69	27.51	positive
E6	Pig tonsils	No Ct	28.77	negative
E7	Pig tonsils	32.84	27.79	positive
E8	Pepton water	No Ct	30.59	negative
E9	Pepton water	No Ct	27.63	negative
E10	Pig tonsils	No Ct	28.04	negative
E11	Pig tonsils	25.67	32.65	positive
E12	Pig tonsils	33.77	27.90	positive
F1	Pig tonsils	19.87	34.76	positive
F2	Pig tonsils	No Ct	28.36	negative
F3	Pig tonsils	31.77	28.35	positive
F4 (negative control)	PCR water	No Ct	28.89	negative

The subject of investigation was the cycle threshold (Ct), which means the number of cycles required for the fluorescent signal to cross the threshold. In Real-Time PCR assay a positive reaction is detected by accumulation of a fluorescent signal [10]. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. The lover the Ct level the greater the amount of target nucleic acid in the sample. The results of investigation proved that F1 sample contained the highest amount of nucleic acid and the fluorescent signal crossed the threshold after 19.87 cycles in the FAM channel. The second sample which contained also a high amount of nucleic acid was E1 sample (Positive Control/Template DNA) in which the fluorescent signal crossed the threshold after 20.96 cycles in the FAM channel.

All the examined Yersinia strains showed FAM cycle threshold (Ct)-values in the range of 19.87–37.63 with 75% of these being between 19.87– 34.00, i.e. strongly positive (Fig. 1). Three strains E6, E10, F2 did not give any fluorescence signal, which was of no consequence since there was no inhibition of the FAM signal, as the fluorescence signal was shown in the VIC channel which meant that the negative results in FAM channel were not caused by inhibition. However, strain E4 with Ct-value 37.63 showed poor amplifiability. We have not sequenced the per gene in this strain but we speculate that this might be due to base pair mutation. This assay will be taken further with regard to

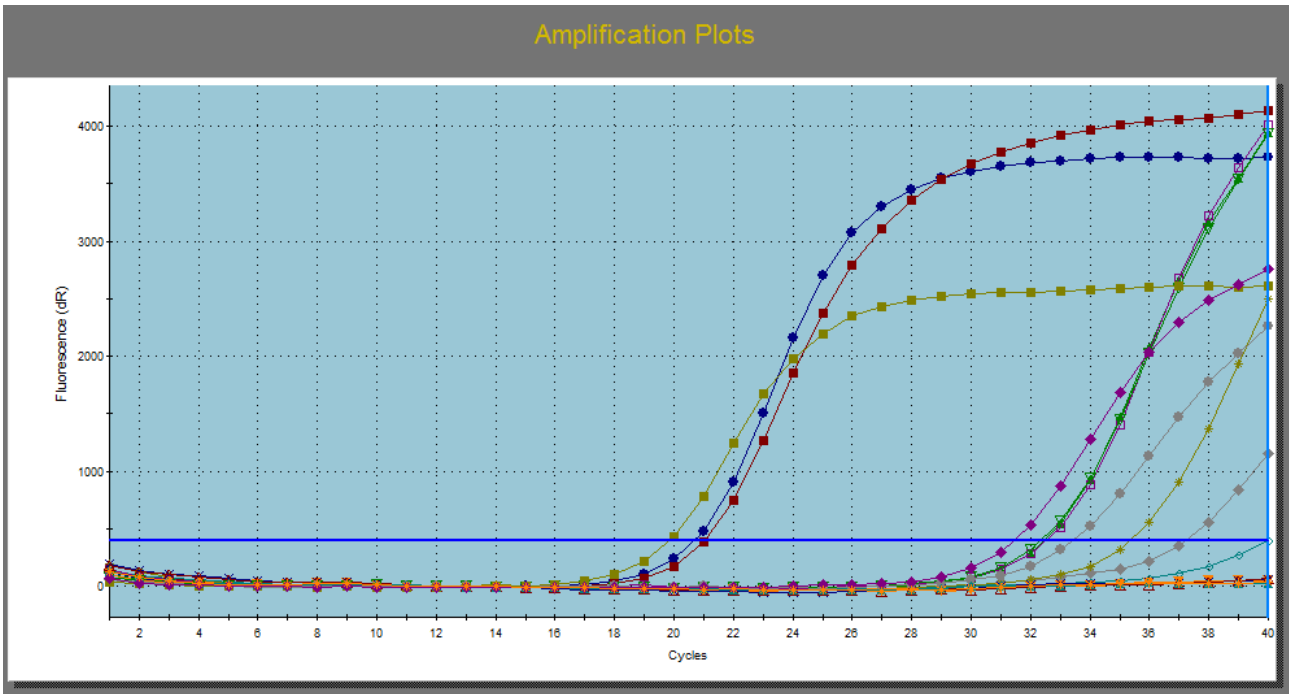

Fig. 1: Fluorescence readings produced from purified DNA from 13 strains of Y. enterocolitica in the Rotor Mx3000P Stratagene Agilent Technologies. The weakest signal was produced by strain E4, which gave a FAM cycle threshold (Ct)-value of 37.64. Three negative controls were included in the assay: two nontemplate controls (NTC) and non-target DNA (NTD)

clinical samples, i.e. tonsil swabs and fecal samples, and further validation in an international collaborative trial [11].

Pathogenic Y. enterocolitica were isolated from 37.4% of the examined samples which means that a great proportion of slaughter pigs coming from one abattoir in Poland transmit this pathogen in their tonsils. However, due to the fact that there is no uniform method for detection, the comparison of achieved results ought to be treated with caution. Taking into account the growing public health concern for human pathogenic Y. enterocolitica in the food chain, significantly more examinations should be carried out in order to obtain knowledge about the occurrence of this pathogen in its main animal reservoir.

In order to examine pathogenic Y. enterocolitica in pigs in a constant way, relevant and reliable detection methods are important because the applied detection method could have an influence on the outcome of the achieved results [2]. To sum up, this study indicates that a significantly high number of slaughter pigs in one abattoir in Poland are carriers of pathogenic Y. enterocolitica in their tonsils. Such a situation leads to the potentially huge source of contamination of edible offal and carcasses during slaughter. As a presence of Y. enterocolitica in food may be a huge risk for human health and life, it is the subject of interest and further investigation.

Acknowledgements

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