Corvinus University of Budapest
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DETECTION, PCR-BASED MOLECULAR IDENTIFICATION AND TYPING OF FOOD SAFETY RELATED BACTERIA

Theses

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1. INTRODUCTION

Food safety is of great importance for both consumers and producers. Increasing demand by consumers for safe, healthy and good quality foods presents a significant challenge for producers, manufacturers and distributors. Meanwhile it remains a challenge for authorities and the participants of the scientific life. As food production has increased significantly in the last few years, the failure of hygienic rules, the improper heat treatment or cooling and other technologies and factors has led to numerous food-borne illnesses. In many cases microbiological contaminations are in the background of these diseases.

Detection and analysis of pathogenic microorganisms by conventional methods have a long history, although these techniques are time consuming and labour-intensive. For this reason rapid methods were developed which can be used in routine analyses as well. Nucleic acid based molecular biological methods are widely used and have increasing popularity. Polymerase chain reaction (PCR) based techniques are essential and regularly applied methods in microbiological laboratories, moreover food microbiological laboratories use this technique more and more frequently. Elaborate nucleic acid based molecular techniques for the identification and typing of pathogenic bacteria from food contribute to the determination of origin and route of transmission of these microorganisms. Numbers of illnesses caused by food-borne pathogenic campylobacters are significant. Ratios of infections in Hungary caused by thermophilic *Campylobacter* species are higher than the average in the European Union, though the number of reported cases shows decreasing tendency. More than ninety-five percentage of human campylobacteriosis is caused by *Campylobacter jejuni*, while for the remaining 5 % strains of *C. coli* are responsible. Unambiguous identification of Campylobacter species is difficult because these campylobacters are fastidious organisms. Differentiation of the species in *Campylobacter* genus is done only by phenotyping methods and the tests resulted in wrong results in many cases. These facts also contributed to the increasing demand on rapid and efficient DNA-based techniques for detection, identification, typing and determination of transmission's rout of *Campylobacter* species.

Strains of Shiga toxin-producing *Escherichia coli* (STEC) which can cause serious illnesses by their numerous virulence factors and toxin production have great importance in public health. Within STEC group detection of strains of enterohaemorrhagic *E. coli* (EHEC) is important because of the haemorrhagic colitis and haemolytic uremic syndrome (HUS) caused by these strains. In the last few years PCR-based diagnostic techniques were developed which targeted different virulence genes such as Shiga toxin, intimine or enterohaemolysin. In

addition to food safety concerns *E. coli* strains are important from food hygienic viewpoint as well as they are indicators of faecal contamination of food. Thus, detection of strains of *E. coli* from food has great importance.

2. OBJECTIVES

The aim of my work was the detection, identification and characterisation of food safety relevant bacteria (*Campylobacter* and *E. coli* strains), thus contributing to the faster detection, determination of diversity and epidemiology of the food originated pathogens.

The tasks and main steps of the work were the following:

1. Isolation of food safety relevant bacteria (*E. coli* and *Campylobacter* spp.) from foods
2. Identification and typing of *E. coli* isolates by molecular biological methods. For the exact identification of the isolates belonging to *E. coli* species and for the detection of O157:H7 serotype development of a duplex PCR method; identification of the isolate by the developed PCR-based technique; and typing of the isolates by RAPD-PCR method.
3. Determination of the effect of enrichment on thermophilic *Campylobacter* species (*C. coli* and *C. jejuni*). Determination of growth curves of the test strains and analysis the growth properties of stains of *C. coli* and *C. jejuni* during the selective enrichment.
4. Identification of *Campylobacter* isolates originated from different sources; and adaptation, elaboration and comparison of applicability of PCR-based molecular techniques on the basis of discrimination ability.

3. MATERIALS AND METHODS

Strains analysed during my work were isolated from foods and from the environment of the foods, while numerous *Campylobacter* and *E. coli* strains were from human and animal sources.

For exact identification and detection of O157:H7 *E. coli* strains a duplex PCR method was elaborated by numerous, Shiga toxin-producing *E. coli* strains. *E. coli* isolates originated from food and the food environment were identified with this elaborated technique. For characterisation of the isolates RAPD-PCR was applied using minisatellite and random primers with different sequences.

For the selective enrichment study growth curves of poultry originated *C. coli* and *C. jejuni* were prepared for each strain using the Bioscreen. To compare the ability of *C. jejuni* and *C. coli* strains to multiply when inoculated in varying proportions in Bolton selective enrichment broth (EN ISO 10272-1:2006) the medium was inoculated with *C. jejuni* /*C. coli* pairs with different proportions of the two species and incubated at 41.5 ºC as it is recommended by the ISO standard. After the incubation time aliquots of cultures were inoculated onto selective agar plates and following the incubation colonies from the agar were identified by hippurate hydrolysis test. Statistical analysis of the results was done by Student's t-test.

Identification of *Campylobacter* isolates originated from different sources was performed by specific primers published in the literature. For testing these primer pairs *Campylobacter* type and reference strains of the four most important thermophilic *Campylobacter* species were used.

Typing of the identified *Campylobacter* isolates was done by six different PCR-based methods. Four enzymes were applied for the restriction of the amplicons in 16S rDNA-RFLP analysis (i), while in case of flagellin encoded *fla*A-RFLP (ii) three restriction enzymes were used. Intergenic sequence (IGS) of *fla*A and *fla*B genes was investigated by denaturing gradient gel electrophoresis (DGGE) analysis (iii). The optimal denaturant concentration was determined by perpendicular DGGE method, and applying the optimal concentration the differences between amplicons with nearly same size were defined by parallel DGGE. Electrophoretic mobility of homoduplex and heteroduplex molecules of the IGS-*fla* was determined in heteroduplex mobility assay (HMA) (iv). Heteroduplexes were formed during the hybridisation of *fla*-IGS sequences of the test and reference strains. For the RAPD-PCR analysis of the isolates (v) three random primers were applied. Based on the results of all six methods dendograms were constructed by softwares and discrimination ability of the techniques was determined by calculating the Simpson's index of diversity. Determining the exact nucleotide sequence of *fla*-IGS region (vi) in case of all isolates the results of DGGE and HMA were estimated.

4. RESULTS

4.1. Isolation and molecular biological analysis of *Escherichia coli* strains

Twenty-two presumptive *E. coli* isolates were collected from foods and from their environment. For the identification of the isolates and detection of strains of O157:H7

serotype a duplex PCR was planned to be elaborated. The PCR was achieved by primer pairs published in the literature, and optimised and tested its applicability by numerous, particularly STEC and EHEC strains. The determined optimal primer concentration was 0.25 μM in case of species specific primers, while in case of the serotype specific primers it was 0.125 μM. The results of the identification of the 22 isolates with the elaborated technique showed that all collected bacteria belonged to the *E. coli* species, but because none of the isolates gave amplicons with the serotype specific primer pair this pathogenic serotype was not presented among the isolates. Characterisation of the isolates was performed with the three most efficient primers. Based on the results of the typing it was established that *E. coli* isolates showed high diversity and their presence, survival and route of transmission are highly significant.

4.2. Effect of enrichment on thermophilic *Campylobacter* strains

In order to determine the competition of thermophilic *Campylobacter* species which are responsible for numerous enteritis growth curves of the eight test strains (four *C. coli* and four *C. jejuni*) were determined in the first step. From these curves early stationary phase of the strains was defined. Investigations to determine the time taken to reach the early stationary phase showed that cultures of both species usually reached this stage after 18 hours of incubation at 41.5 ^{o}C, although there were considerable variations in the lag time. Enrichment studies performed with cells of different *C. coli* and *C. jejuni* strains from the same growing stage showed that *C. coli* isolates were able to multiply better in Bolton selective enrichment broth. *C. jejuni* was isolated in higher number only in one combination of the strains. To confirm that the achieved ratios were different from the inoculated ones the Student t-test was applied. According to the results of the statistical analysis the p-values were highly significant for each of the four comparisons, thus, the ratios observed at the beginning and at the end of the enrichment were significantly different. This implies that during the enrichment the isolate of *C. coli* always multiplied better than the strain of *C. jejuni*. As five or fewer colonies of campylobacter are often picked from the selective agar when examining foods or other samples, these results indicate that if the sample contains a mixture of *C. jejuni* and *C. coli, C. coli* is much more likely to be isolated than *C. jejuni*, and our conclusions concerning the predominant species might not be correct.

4.3. Analysis of *Campylobacter* isolates by PCR-based molecular methods

Specific primer pairs published in the literature were chosen for the identification of *Campylobacter* isolates originated from different sources. Based on the results of the identification from the 55 isolates 24 belonged to *C. jejuni* species, while 31 were isolates of *C. coli* species. The 16S rDNA-RFLP method used for the analysis of the identified isolates did not prove to be suitable for the characterisation of *C. jejuni* and *C. coli* strains, which was also confirmed by its very low discrimination index. PCR-RFLP analysis of FlaA protein encoding *fla*A gene was utilized for typing of the tested isolates. The Simpson's index of diversity was 0.989 of *fla*A-RFLP method. For parallel DGGE analysis of *fla*-IGS region the 20-40 % denaturing concentration proved to be applicable, and based on the results of DGGE it was established that comparing the *fla*-IGS DGGE technique with *fla*A-RFLP the later method had better discrimination power. In the case of the heteroduplex mobility assay most of the isolates formed heteroduplex molecules with the type/reference strains which refers to the sequence polymorphism of the 180-200 base pair DNA sequences. Diversity index of the heteroduplex mobility assay was 0.872, so the method is able to characterise isolates at strain level, but similarly to the DGGE analysis it is not applicable for species identification. RAPD-PCR analysis using three different primers was able not only to type the strains but to differentiate the two tested species of *Campylobacter* genus. Comparing the results of direct sequencing of the *fla*-IGS region with results of DGGE and heteroduplex mobility assay it was established that the DGGE and HMA are good techniques for detection of sequence differences within shorter gene sequences, but their applicability for typing is limited.

According to my knowledge in the literature no data can be found concerning the comparison of different PCR-based typing methods in case of Hungarian *Campylobacter* isolates, that is why results of my work can be considered as novel in typing of *C. jejuni* and *C. coli* strains in Hungary.

4.4. Possibilities of utilisation and improvement of the results

Pathogenic *Campylobacter* species can be characterised with low infectious dose, but their detection from complex food matrixes or clinical samples in presence of competing microbiota can be achieved by selective enrichment. Nevertheless with respect to epidemiological and zoonotic analyses it is essential that from a sample containing strains of

C. coli and *C. jejuni* which species could be detected if selective enrichment had to be used due to low cell number. Further studies including food samples, competitive microbiota and more *C. coli*/*C. jejuni* strains could analyse this phenomenon '*in vivo*'.

Identification of O157:H7 and O157:H^- serotypes among enterohaemorrhagic *E. coli* (EHEC) strains is important because of the serious illnesses these bacteria can cause. Application of the elaborated duplex PCR method for the detection of *E. coli* species as well as EHEC and some Shiga toxin-producing *E. coli* (STEC) strains in one PCR reaction can contribute to the detection of fresh faecal contamination and to the determination of food safety status of the sample in food industry and in diagnostic laboratories. As infectious dose of EHEC strains are low and possible contamination of food threaten the consumers' health rapid and reliable detection of presence or absence of pathogens is particularly important. Designing specific probes for the target sequences (*mal*B and *hly*A) and optimising further the technique a duplex real-time PCR method could be developed by which detection of *E. coli* strains could be performed in real time.

Identification, typing and determination of rout of transmission with proper molecular biological methods in the case of thermophilic *Campylobacter* species is an important task. Exact identification of strains causing human illnesses is significant in clinical microbiology, since types of antibiotics suitable for the treatment of diseases depend on the species caused the illness. Furthermore differentiation of *C. coli* and *C. jejuni* species and strains of the two species is significant because certain strains of *C. jejuni* can cause serious neurological disorders, while strains of *C. coli* can be less related to serious diseases. It is also important to determine the types of foods by which the two species can enter into the food chain, because more information is obtained in terms of zoonoses and this helps the protection against these microorganisms. It would be important to develope reliable and rapid PCR-based techniques for the detection of other thermophilic campylobacters because in absence of these methods identification and characterisation of *Campylobacter* species other then *C. coli* and *C. jejuni* – mainly in routine diagnoses – is difficult.

5. NEW SCIENTIFIC ACHIEVEMENTS

(1) A duplex PCR method has been developed for the detection of *E. coli* species and its O157:H7 serotype in one reaction, in which the rapid and exact identification of the isolates can be performed with two sets of primers. The recent faecal contaminant *E. coli*,

the food safety relevant EHEC (enterohaemorrhagic *Escherichia coli*) and some other important STEC (Shiga toxin-producing) strains can be detected by this method.

(2) Comparing the multiplication ability of different *Campylobacter jejuni* and *C. coli* strains during selective enrichment in Bolton broth it has been established that in majority of the cases strains of *C. coli* were able to multiply better than strains of *C. jejuni*. Thus, after selective enrichment identification of the isolates could lead to incorrect results if the two species occur in the examined sample together.

(3) Significant differences were found among *Campylobacter* isolates by comparing the intergenic sequences of *fla*A and *fla*B genes. It has been showed that behind sequence polymorphisms single nucleotide biases and some tens of nucleotide insertions and deletions are responsible. The denaturing gradient gel electrophoresis analysis and the earlier not applied heteroduplex mobility assay of the intergenic sequence have been able to discriminate the isolates with the same efficacy. It refers to the appropriate sensitivity of the methods in case of the polymorphism analysis within a given gene.

(4) The PCR-based methods for typing of *Campylobacter coli* and *C. jejuni* isolates were compared by determining their Simpson's index of diversity. It has been established that from the aspect of discrimination ability the most appropriate technique for characterisation of both species is the RAPD-PCR analysis. It is followed by the RFLP analysis of the *fla*A gene, which proved to be a good technique for typing of *C. coli* and *C. jejuni* strains. Based on the result obtained sequence typing of the *fla*-IGS sequence has prominent discrimination ability in case of *C. jejuni*, while it has acceptable discrimination power in case of *C. coli*. For that reason it is recommended for the practice as a usually applicable typing method. The *fla*-IGS DGGE (denaturing gradient gel electrophoresis) and the *fla*-IGS HMA (heteroduplex mobility assay) performed the poorest, but still exhibited a satisfactory discrimination index.

(5) Evaluated the results of the typing methods by principle component analysis it has been established that although the molecular typing methods did not result in specific patterns suitable for the identification of the isolates belonged to *C. coli* and *C. jejuni* species, isolates of the two species clustered into distinct groups by the statistical analysis. It indicates that based on the sequences analysed by typing methods strains of one species showed major similarity with each other when compared with strains of the other species.

6. PUBLICATIONS

JOURNALS

In journals with impact factor

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