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Annealing Temperature of 55°C and Specificity of Primer Binding in PCR Reactions

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Abstract

In our study, we used PCR to clone *papA*, *papEF*, *papG* and *F17G* genes of *Escherichia coli* isolated from faecal samples of dogs with diarrhoea. Annealing temperature of 55°C was used in the PCR. Nucleotide sequence analysis of 26 cloned PCR products showed that in PCRs with *papA* primers, six out of eight obtained PCR products were false due to non-specific binding of the forward primer on both DNA strands; in PCRs with the *papEF* primers, all seven obtained PCR products originated from specific binding of the forward primer on the 3' → 5' DNA strand and non-specific binding of the forward primer on the 5' → 3' DNA strand; and in PCRs with the *F17G* primers, four out of eight obtained PCR products were false due to non-specific binding of forward and reverse primer. The anticipated annealing sites for non-specific primer binding in analysed nucleotide sequences are presented. In the case of PCR products obtained with *papG*-specific primers, all PCR products were amplifications of the *papG* sequence. When the annealing temperature of *papA* PCRs was raised to 60°C, all obtained PCR products were amplifications of the correct DNA sequences.

Keywords: primer binding, annealing temperature, sequence analysis, *Escherichia coli*, adhesin, P-fimbriae, F17-fimbriae

1. Introduction

Since polymerase chain reaction (PCR) was invented in the mid-1980s, it has made its way into all molecular biology, genetic, microbiology or biochemistry laboratories, where it is, due to its simplicity and efficiency, used in a very wide range of (PCR)-based techniques and applications [1, 2]. In just a few hours with a certain amount of cycles consisting of three simple steps—DNA denaturation, annealing of primers and extension [2]—the desired DNA sequence is multiplied about a million fold [3]. The crucial step in PCR is the annealing of primers, where the annealing temperature determines the specificity of primer annealing. The annealing temperature of a standard PCR protocol is either 55°C [2, 3] or 60°C [4]. The chosen temperature depends on the strand-melting temperature of the primers and the desired specificity. For greater stringency higher temperatures are recommended [2].

PCR is very often used to amplify specific DNA fragments that are later cloned as inserts in plasmid vectors and used then in subsequent experiments. Examples of

such subsequent experiments are nucleotide sequencing, in order to determine the nucleotide sequence of the insert or in vitro transcription, and translation, in order to obtain a certain protein.

In our experiments, the aim was to determine the nucleotide sequence of several fimbrial genes from different *Escherichia coli* (*E. coli*) strains isolated from faecal samples of dogs with diarrhoea. The genes of interest were *papA*, *papG*, *papEF* of the P-fimbriae and *F17G* of the F17-fimbriae. Therefore, from a collection of 24 clinical haemolytic *E. coli* strains from faecal samples of dogs with diarrhoea [5], genomic DNA was isolated and used as the matrix DNA to amplify these genes of interest with gene-specific primers with PCR. Further, the obtained PCR products were cloned into a TA cloning vector, and the nucleotide sequence was determined.

2. *Escherichia coli*

Escherichia coli is one of the best studied organisms. It belongs to the family of *Enterobacteriaceae*. It is a Gram-negative rod-shaped bacterium, non-sporulating, non-motile or motile by peritrichous flagella, chemoorganotrophic, facultative anaerobic, producing acid from glucose, catalase positive, oxidase negative and mesophilic [6].

It is a well-known commensal bacterium that is part of the gut microbiota of humans and other warm-blooded organisms. However, also pathogenic strains of *E. coli* do exist and can cause a variety of intestinal and extraintestinal infections in humans and many animal hosts. *E. coli* is considered to be one of the most important pathogens; it is the most frequently isolated species in clinical microbiology laboratories [7]. Intestinal pathogenic *E. coli* (IPEC) strains, also called diarrhoeagenic *E. coli* (DEC) strains, are divided into six different well-described categories, i.e. pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [8]. DEC causes diarrhoea syndromes that vary in clinical presentation and pathogenesis depending on the strain's pathotype [7]. *E. coli* strains involved in diarrhoeal diseases are one of the most important among the various etiological agents of diarrhoea [9]. The extraintestinal pathogenic *E. coli* (ExPEC) strain group is comprised of different *E. coli* associated with infections of extraintestinal anatomic sites [10]. Traditionally, the ExPEC isolates are separated into groups determined by disease association, i.e. uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC), naming the most important ExPEC groups. But ExPEC strains are also implicated in infections originating from abdominal and pelvic sources (e.g. biliary infections, infective peritonitis and pelvic inflammatory disease) and also associated with skin and soft tissue infections and hospital-acquired pneumonia [11]. Due to its genotypic and phenotypic diversity, *E. coli* is known as the paradigm for a versatile bacterial species [12].

The pathogenic strains possess specialised virulence factors such as adhesins, toxins, iron acquisition systems, polysaccharide coats and invasins that are not present in commensal strains [7].

3. Adhesins

Adhesins play a very important role in the host-microbe interactions, as they convey the adherence to the epithelial host's cells, surface structures or molecules.

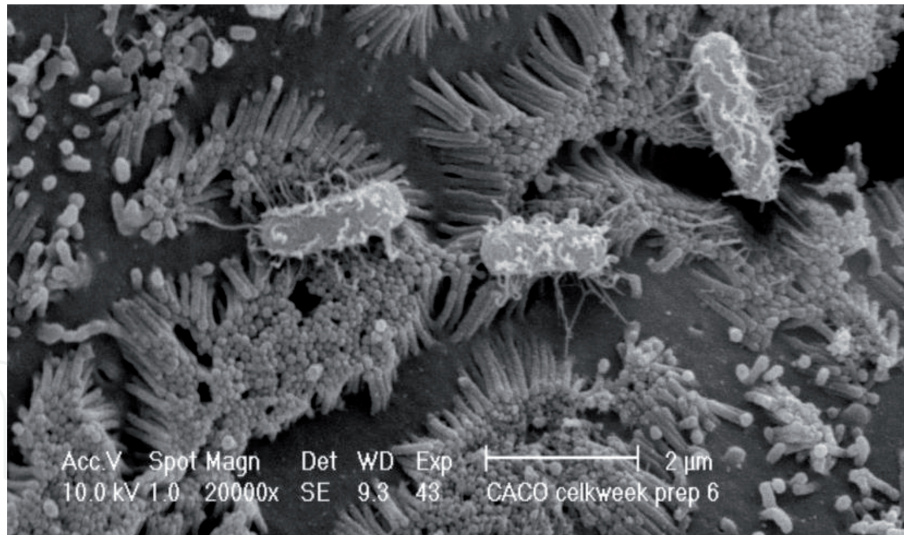


Figure 1.
 Scanning electron microscopy of *Escherichia coli* strain 963 adhering to 19-day-old Caco-2 cells [5]. The fimbrial structures on the bacterial surfaces are promoting the bacterial adherence to receptors on host cells.

Adhesion is the essential first step for most commensal and pathogenic bacteria in order to colonise and persist within the host [13]. While adhesion to abiotic surfaces is usually mediated by non-specific interactions, adhesion to biotic surfaces typically involves specific receptor-ligand interaction [14]. Adhesins are structures on the bacterial surface that help the bacteria to bind to receptors on host's cells (**Figure 1**).

Adhesins are not just involved in adherence but also in bacterial invasion, survival, biofilm formation, serum resistance and cytotoxicity [15]. Moreover, they are also involved in bacterial motility and DNA transfer [13]. They differ in their architecture and receptor specificities. Types of adhesin vary depending on the Gram nature of bacteria [15].

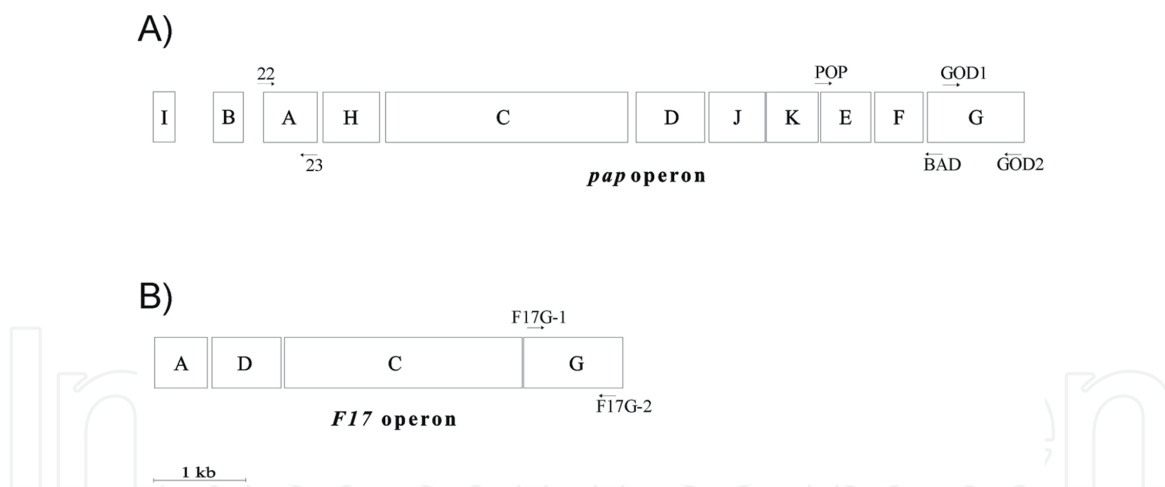
Adhesins are among the most important virulence-associated properties of *E. coli*, as they are the main virulence factors of bacteria needed in bacterial colonisation. There are two types of bacterial adhesins: fimbrial and afimbrial [16].

Fimbrial adhesins, i.e. fimbriae, are rodlike structures with a diameter of 5–7 nm. Each fimbria consists out of several hundred copies of a protein, whose generic name is 'major subunit', and other proteins, present in one or a very few copy number and called 'minor subunits' that are positioned either at the basis or at the top of the fimbriae or intercalated between the 'major subunits' [16]. Fimbriae can be even longer than 1 μm [13]. On the bacterial surface of wild-type *E. coli* strains, there are around 500 fimbriae [17]. P-fimbriae and F-17 fimbriae belong to the fimbrial adhesins.

Non-fimbrial adhesins are monomeric or trimeric structures that decorate the surface of bacteria. These adhesins are anchored to the surface of the outer membrane and due to their small size, the size of non-fimbrial adhesins is approximately 15 nm, allow an intimate contact between the bacterial cell surface and specific substrates. One of the major classes of non-fimbrial adhesins is autotransporter adhesins [13].

3.1 P-fimbriae

P-fimbriae are the most extensively studied adhesins. They are also the first virulence-associated factor found among uropathogenic *E. coli*. These fimbriae bind to Gal(α1–4)Galβ moieties of the membrane glycolipids on human erythrocytes

**Figure 2.**

Scheme of *pap* and *F17* operon and annealing sites of the used primers. Genes in the operon are presented as boxes. The positions of used primers to amplify the studied genes are marked with arrows.

(A) Scheme of *pap* operon. The scheme of *pap* operon was drawn based on the GenBank deposited nucleotide sequence X61239.1 [20] and (B) scheme of *F17* operon. The scheme of *F17* operon was drawn based on the GenBank deposited nucleotide sequence L77091.1 [26].

of the P blood group and on uroepithelial cell fimbriae [18]. Further receptors for P-fimbriae are present on erythrocytes from pigs, pigeon, fowl, goats and dogs but not on those from cows, guinea pigs or horses [19]. These fimbriae are encoded in the *pap* operon, consisting of 11 different genes (see **Figure 2A**): *papA* (558 bp), *papB* (315 bp), *papC* (2511 bp), *papD* (720 bp), *papE* (522 bp), *papF* (504 bp), *papG* (1008 bp), *papH* (588 bp), *papI* (234 bp), *papJ* (582 bp) and *papK* (537 bp) [20].

The product of the *papA* gene is the major subunit protein A (19.5 kDa) [19]. In *papB* a regulatory protein (13 kDa) is encoded. PapB is necessary for the activation of the *papA* expression [21]. PapC (80 kDa) is located in the outer membrane and forms the assembly platform for fimbrial growth. PapD (27.5 kDa) is present in the periplasmic space and is involved in the translocation of fimbrial subunits across the periplasmic space to the outer membrane prior to assembly. PapE (16.5 kDa), PapF (15 kDa) and PapG (35 kDa) are minor fimbrial components. PapG is the adhesin molecule conferring the binding specificity [19]. PapH (20 kDa) terminates fimbrial assembly and helps anchor the fully grown fimbriae to the cell surface [22]. PapI (12 kDa) is another regulatory protein involved in *papA* expression due to activation of *papB* promoter [21]. PapJ (18 kDa) is a periplasmic protein required to maintain the integrity of P-fimbriae [23]. PapK (20 kDa) regulates the length of the tip fibrillum and joins it to the rod [24].

Many variants of P-fimbriae exist. PapA molecules from different P-fimbrial serovariants have a high degree of similarity at the N and C termini, while the central portions of PapA exhibit a great variation in the primary structure. This central part of PapA is hydrophilic and exposed and hence under selective pressure from the host immune system. Substantial heterogeneity is also between different minor fimbrial subunits (PapE, PapF and PapG) [19]. In addition also P-fimbria-related fimbriae, the so-called Prs-fimbriae, exist. Prs-fimbriae are encoded in the *prs* (*pap*-related sequence) operon [18].

3.2 F17-fimbriae

F17-fimbriae are found on pathogenic *E. coli* strains, isolated from infections in domestic animals. They are mainly detected on bovine and ovine *E. coli* associated with diarrhoea or septicaemia but also on *E. coli* from other hosts, including humans. The F17 adhesin binds to N-acetyl-D-glucosamin receptors of bovine intestinal cells;

however, F17 subtypes were also found to bind to N-acetyl-D-glucosamin receptors of human uroepithelial and intestinal cells [25]. The F17-fimbriae are encoded in the *F17* operon, consisting of four genes: *F17A* (546 bp), *F17D* (723 bp), *F17C* (2469 bp) and *F17G* (1035 bp) (see **Figure 2B**) [26].

F17A protein (20 kDa [25]) is the structural component of the F17-fimbriae (major subunit protein). The F17A protein is homologous to PapA protein of the P-fimbriae [27]. F17C protein (90 kDa) probably functions as a base protein on which the fimbrial subunits are polymerised. F17D protein (28 kDa) has a close homology to the PapD protein of the P-fimbriae [28]. It functions as the periplasmic transport protein [29]. F17G protein (36 kDa [25]) is the minor fimbrial component required for the binding of the F17-fimbriae to its receptor on the host cell [30].

Several variants of F17-fimbriae exist. The diversity is based on differences in *F17A* and *F17G* genes. The variant of F17-fimbriae found in humans is designated as G-fimbriae, encoded in the *gaf* operon [25].

4. Materials and methods

4.1 Bacterial strains, growth media and conditions

The analysed 24 clinical haemolytic *E. coli* strains [5] originated from dogs with diarrhoea and were isolated at the Veterinary Microbiological Diagnostics Centre of Utrecht University, the Netherlands. Some more details about the strains are given in **Table 1**. As positive control strains, a dog uropathogenic *E. coli* strain (strain 1473) and a cattle mastitis *E. coli* strain (strain E5) from Wim Gaastra's *E. coli* collection were used [31].

All used bacterial strains were stored at -80°C as a suspension in a 1:1 mixture of L-broth and glycerol as published by Garcia et al. [32]. The strains were grown overnight on LB plates and in liquid LB medium at 37°C . When grown in liquid LB medium, the flasks with the bacterial culture were incubated with aeration.

4.2 Isolation of chromosomal DNA

Chromosomal DNA was isolated from all 24 clinical haemolytic *E. coli* strains [5] and strains used for positive controls [31] using a slightly modified protocol based on the protocol of miniprep of bacterial genomic DNA published by Ausubel et al. [33]. To summarise, 2 ml of an overnight bacterial culture was centrifuged for 2 min at 14,000 rpm at room temperature. The obtained bacterial pellet was resuspended in 567 μl of buffer TE and 6 μl of 0.5 M EDTA. The suspension was incubated for 15 min at -80°C . Following the incubation at -80°C , the suspension was thawed, and 10 μl of 25 mg/ml proteinase K solution was added. The suspension was mixed thoroughly, and 30 μl of 10% SDS was added to the suspension and mixed thoroughly again. A 2-hour incubation at 37°C followed, and then 100 μl of 5 M NaCl was added to the suspension and mixed thoroughly. Next 80 μl of CTAB/NaCl was added, mixed thoroughly again and incubated at 65°C for 10 min. After the incubation the suspension was treated with 200 μl of chloroform/isoamyl alcohol and centrifuged for 5 min at 14,000 rpm at room temperature. The aqueous supernatant was transferred to a fresh microcentrifuge tube and treated with 100 μl of phenol/chloroform/isoamyl alcohol and centrifuged for 5 min at 14,000 rpm at room temperature. The aqueous supernatant was transferred to a fresh microcentrifuge tube, and the DNA in the aqueous supernatant was precipitated with addition of 0.6 volume of isopropanol. The precipitated chromosomal DNA was transferred to a fresh microcentrifuge tube containing 100 μl of 70% ethanol. The precipitated

Strain number	Dog's age	Clinical signs	Serotype	MSHA	MRHA
297	Unknown	Unknown	O78K?	E, P, C	C
333	Young dog	Chronic diarrhoea	O6K53	E, P, C, O	P, O
366	Unknown	Unknown	NT	B, E, P, C, O	B, E, P, O
375	Pup	Sepsis	O20K?	None	P
380	Unknown	Unknown	O6K-	B, E, P, C, O	P, C, O
442	Pup	Sepsis diarrhoea	O4K2	B, P, C, O	E, P, C, O
450	Pup	Sepsis diarrhoea	O42K-	E, P, C	None
467	5 months	Chronic diarrhoea	O6K1	E, P, C, O	P, C
479	Pup	Sepsis diarrhoea	O23K1	B, E, P, C	P, C, O
503	Unknown	Unknown	O42K-	C, O	None
555	Pup	Sepsis diarrhoea	O4K53	E, P, C, O	O
567	3 months	Recurrent diarrhoea	O4K?	E, P, C, O	P, C, O
727	3 months	Unknown	O139K?	E, P, C	P
734	18 months	Chronic diarrhoea	O4K?	E, P, C, O	E, P, C, O
740	4 years	Chronic diarrhoea	O25K13	E, P, C, O	P, C, O
759	12 months	Chronic diarrhoea	O4K?	P, O	P, O
857	9 months	Chronic diarrhoea	NT	P, C, O	P, O
897	Unknown	Chronic diarrhoea	O6K53	B, E, P, C, O	B, E, P, C, O
912	Old dog	Chronic diarrhoea	O4K-	E, P, C, O	E, P, C, O
958	Pup	Sepsis diarrhoea	NT	B, C, O	None
963	Unknown	Chronic diarrhoea	O4K?	B, E, P, C	B, E, P, C
996	4 months	Unknown	O4K?	E, P, C, O	O
1205	Unknown	Chronic diarrhoea	O4K-	E, P, C, O	E, P, C, O
1237	3 months	Bloody diarrhoea	O2K1	B, E, P, C, O	B, E, P, C, O

MSHA is an abbreviation of mannose-sensitive haemagglutination, and MRHA is an abbreviation of mannose-resistant haemagglutination. The erythrocytes are abbreviated as follows: B, bovine erythrocytes; E, equine erythrocytes; C, canine; O, ovine; P, porcine. NT, non-typable.

Table 1.
Characteristics of the 24 studied *E. coli* strains [5, 31].

DNA in 70% ethanol was pelleted with centrifugation (10 min at 14,000 rpm at room temperature). The 70% ethanol was then removed and the chromosomal DNA pellet air-dried at 37°C. Finally the chromosomal DNA pellet was dissolved in 100 µl of sterile distilled water.

4.3 PCR mixtures for PCR amplification of P- and F17-fimbrial genes

One µl of the isolated chromosomal DNA was used in 50 µl PCR mixtures consisting of 20 pmol of each primer, 0.2 mM dNTP mixture and 0.625 U of *Taq*-polymerase in PCR buffer [5]. In PCRs for P-fimbrial genes for positive control samples, the isolated chromosomal DNA of the dog uropathogenic *E. coli* strain (strain 1473) was used. In PCRs for F17-fimbrial gene for positive control samples, the isolated chromosomal DNA of the cattle mastitis *E. coli* strain (strain E5) was used. In all PCRs for the negative control, sterile distilled water was used [31].

4.4 Primers and PCRs used to amplify P- and F17-fimbrial genes

Primers used in the PCRs to amplify the studied genes are listed in Table 2.

PCR for gene(s)	Primers (name, sequence of the primer 5' → 3')	Tm (°C) of primers
<i>papA</i>	22	84.1
	ATGATGAATTTCGGTTATTGCCGGTGCGG	
	23	68.3
<i>papEF</i>	CTGAGAATTCAGGTTGAAATTTCGC	
	POP	63.8
	CCACGTTTGAATTGACATATCG	
	BAD	72.9
<i>papG</i>	CCGTAGCAATGCCCCGGGC	
	GOD1	65.5
	ATGGTACCCAGCTTTGTTATTTTCC	
	GOD2	62.4
<i>F17G</i>	TGGCAATATCATGAGAAGCTTT	
	F17G-1	72.4
	CAGGCGGCAGTTTCATTATTGGC	
	F17G-2	76.4
	CCGGACTGAGGGTGACGTTTCCGT	

Table 2.
Primers and their melting temperatures (Tm) used to amplify the studied genes.

Predicted primer annealing sites of the used primers on the target operons are shown in Figure 2.

The PCR amplification in all the reactions for all studied genes was carried out in the following steps: heating at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and the final extension for 10 min at 72°C.

The expected sizes of PCR products were determined with the ‘Primer-BLAST’ online tool (data set nr organism *Escherichia coli*) on the Internet page of the National Center for Biotechnology Information, US National Library of Medicine (<http://www.ncbi.nlm.nih.gov>) as follows: *papA*—552 bp (GenBank deposited nucleotide sequence LR134092.1), 555 bp (GenBank deposited nucleotide sequence CP025703.1), 534 bp (GenBank deposited nucleotide sequence CP018957.1), 564 bp (GenBank deposited nucleotide sequence CP029579.1) and 561 bp (GenBank deposited nucleotide sequence CP024886.1); *papEF*—1372 bp (GenBank deposited nucleotide sequence CP027701.1), 1373 bp (GenBank deposited nucleotide sequence CP028304.1), 1367 bp (GenBank deposited nucleotide sequence CP026853.1) and 1371 bp (GenBank deposited nucleotide sequence LR134238.1); *papG*—1000 bp (GenBank deposited nucleotide sequence CP026853.1) and 1003 bp (GenBank deposited nucleotide sequence M20181.1); and *F17G*—888 bp (GenBank deposited nucleotide sequence AF055313.1) and 885 bp (GenBank deposited nucleotide sequence CP001162.1).

4.5 Agarose gel electrophoresis

Samples of isolated chromosomal DNA (5 µl of isolated chromosomal DNA and 1 µl of 6 × loading dye) were subjected to analysis with agarose gel electrophoresis using

1% of agarose gels with 0.5 µg/ml ethidium bromide, run in 0.5 × TBE electrophoresis buffer. Samples of obtained PCR products (25 µl of PCR products, 5 µl of 6 × loading dye) were subjected to analysis with agarose gel electrophoresis using 1% of agarose gels with 0.5 µg/ml ethidium bromide, run in 1 × TAE electrophoresis buffer. Used protocols for agarose gel electrophoresis were based on Sambrook et al. [34]. For DNA ladder the lambda bacteriophage DNA digested with the restriction endonuclease *Pst*I was used.

4.6 Cloning of PCR products and DNA sequencing of cloned PCR products amplified in PCRs for P- and F17-fimbrial genes

Cloning of PCR products and DNA sequencing of cloned PCR products obtained in the PCRs for P- and F17-fimbrial genes was done as described by Starčič et al. [5]. In short, obtained PCR products were cut out of the agarose gel, cleaned with the GeneClean II Kit, inserted into the TA cloning vector pMOSBlue and then transformed to electrocompetent *E. coli* pMOSBlue cells. Subsequently, the plasmid DNA was isolated from pMOSBlue cells using the FlexiPrep Kit, and the nucleotide sequence was determined with the dideoxynucleotide chain termination method using an automated laser fluorescence sequencer. All procedures were performed according to the manufacturers' protocols.

4.7 Analysis of cloned nucleotide sequences of the PCR products amplified in PCRs for P- and F17-fimbrial genes

Sequence analysis of the cloned fragments, originated from PCR products obtained in PCRs for P- and F17-fimbrial genes, was performed with the computer program BLAST on the Internet page of the National Center for Biotechnology Information, US National Library of Medicine (<http://www.ncbi.nlm.nih.gov>) searching for homology in the GenBank nr database.

5. Results

5.1 Analysis of cloned nucleotide sequences from PCRs with primers specific for *papA*

An annealing temperature of 55°C was used in the PCRs for the amplification of the *papA* gene with primers 22 and 23. The obtained PCR products were all of the expected size (around 600 bp). However, the nucleotide sequence analysis of the eight obtained cloned PCR products revealed that six clones harboured false, non-*papA* inserts. Four of these false clones derived from amplification of part of methylisocitrate lyase gene, and two clones derived from amplification of part of the RNA-binding protein Hfq gene and part of the GTPase HflX gene, as revealed by BLAST analysis. In both cases even though both primers, forward primer 22 and reverse primer 23, were added to the PCR mixture, the primer 22 was used as the forward but also the reverse primer. Further nucleotide analysis revealed that in the case of the amplification of part of the methylisocitrate lyase gene, the forward primer annealed downstream from the c348059 position of the 3' → 5' strand and reverse primer upstream of the 348539 position on the 5' → 3' DNA strand of the *E. coli* K-12 MG1655 sequence as deposited in the CP025268.1 nucleotide sequence [35]. The anticipated annealing sites for non-specific *papA*-primer binding in this case are presented in **Figure 3**.

In the case of the amplification of part of the RNA-binding protein Hfq gene and part of the GTPase HflX gene, the forward primer annealed downstream from

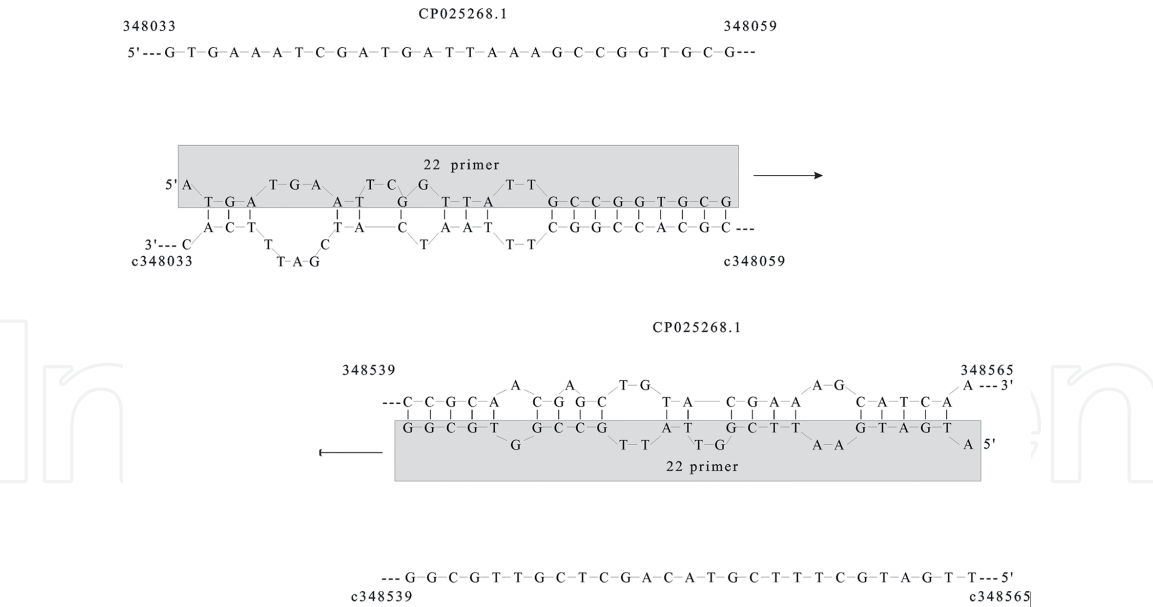


Figure 3. Anticipated annealing sites for non-specific *papA*-primer binding in the methylisocitrate lyase gene. The shown sequences are enumerated according to the CP025268.1 GenBank deposited sequence [35]. The sequence and the complement chromosomal sequence are given. For the forward primer annealing site, the sequence from 348033 to 348059 nt is shown, and for the reverse primer annealing site, the sequence from 348539 to 348565 nt is shown. The primer sequence is in the grey box. The arrows mark the direction of DNA elongation in the PCR. The methylisocitrate lyase gene is positioned in the deposited sequence from 347733 to 348623 nt.

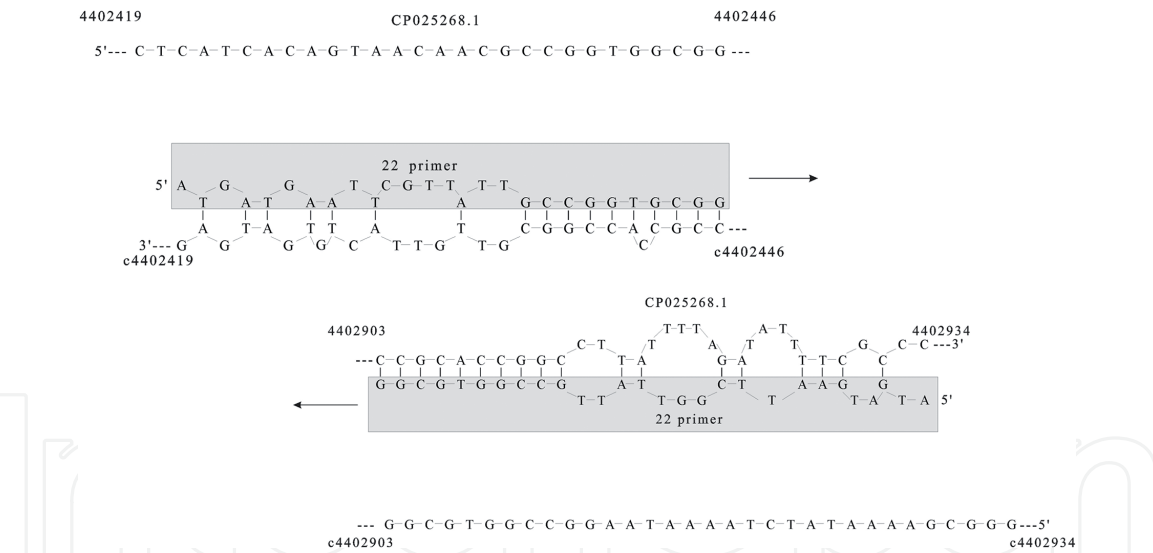


Figure 4. Anticipated annealing sites for non-specific *papA*-primer binding in the RNA-binding protein Hfq gene (forward primer) and GTPase HflX gene (reverse primer). The shown sequences are enumerated according to the CP025268.1 GenBank deposited sequence [35]. The sequence and the complement chromosomal sequence are given. For the forward primer annealing site, the sequence from 4402419 to 4402446 nt is shown, and for the reverse primer annealing site, the sequence from 4402903 to 4402934 nt is shown. The primer sequence is in the grey box. The arrows mark the direction of DNA elongation in the PCR. The RNA-binding protein Hfq gene is positioned in the deposited sequence from 4402214 to 4402522 nt, and the GTPase HflX gene is positioned from 4402598 to 4403878 nt.

the c4402446 position on the 3' → 5' DNA strand, and the reverse primer annealed upstream from the 4402903 position on the 5' → 3' DNA strand of the *E. coli* K-12 MG1655 sequence as deposited in the CP025268.1 nucleotide sequence [35]. The anticipated annealing sites for non-specific *papA*-primer binding in this case are presented in **Figure 4**.

5.2 Analysis of cloned nucleotide sequences from PCRs with primers specific for *papEF*

In the PCRs for the *papEF* amplification, also the annealing temperature of 55°C was used. Seven PCR products, all of the expected size, of around 1400 bp, were cloned, and the obtained insert sequences were analysed. All seven clones harboured the amplified *papEF*-related sequence, the *prsEF* sequence of the *Prs-fimbriae* (GenBank X61238.1 [36]); however, in all seven cases, only the forward POP primer annealed to the correct complementary sequence from c27 to c48 nt on the 3' → 5' DNA strand of the X61238.1, while the reverse primer was not as expected the BAD primer but again the POP primer, which annealed at another partially complementary sequence of the *prsEF* gene from 1357 upstream on the 5' → 3' DNA strand. Further BLAST analysis showed that the BAD primer has only a partial complementary region of nine nucleotides at the position 3229 to 3237 in the 5' → 3' DNA strand and at the position c3238 to c3230 in the 3' → 5' strand of the X61238.1 sequence. The anticipated annealing sites of POP primer on the analysed X61238.1 nucleotide sequences are presented in **Figure 5**.

5.3 Analysis of cloned nucleotide sequences from PCRs with primers specific for *papG*

In the PCRs for the *papG* amplification, also the annealing temperature of 55°C was used. Three PCR products, all of the expected size, of around 1000 bp, were obtained and cloned, and the obtained insert sequences were analysed. All three clones harboured the expected *papG* sequence. In all three PCR amplifications, both primers, the forward GOD1 and reverse GOD2 primer, annealed at the expected positions. The anticipated annealing sites for specific *papG*-primer binding

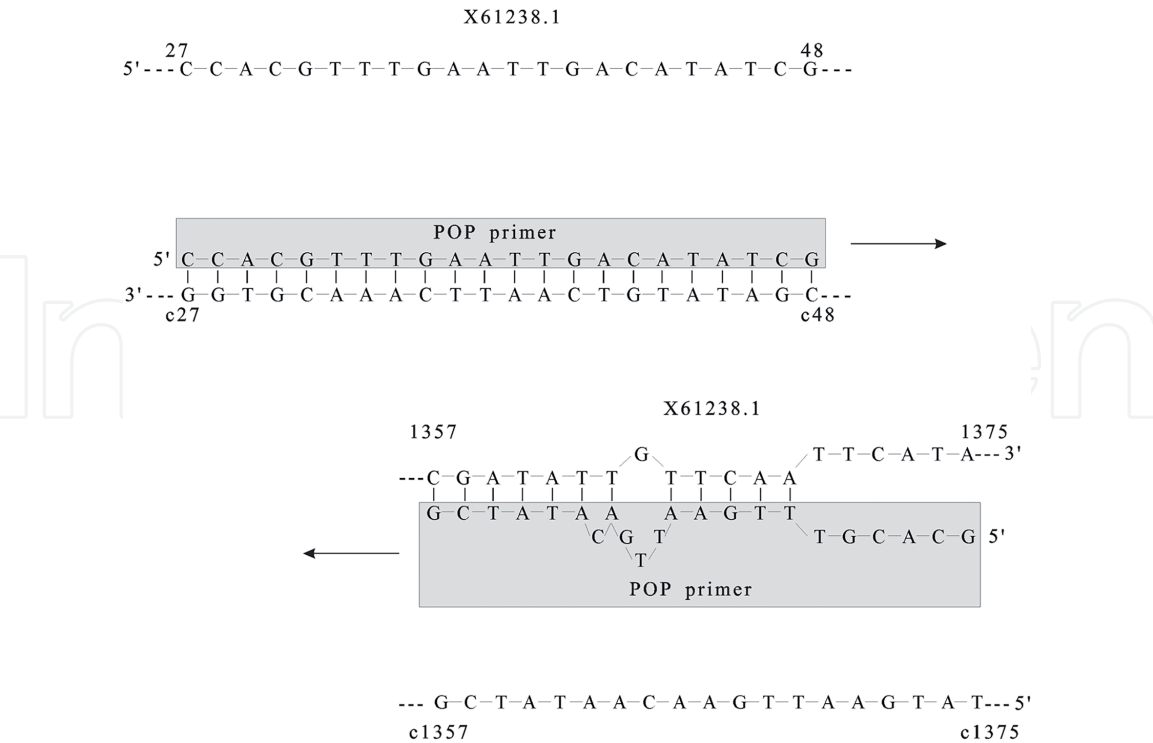


Figure 5. Anticipated annealing sites of primer POP in the *prsEF* sequence. The shown sequences are enumerated according the X61238.1 GenBank deposited sequence [36]. The sequence and the complement chromosomal sequence are given. For the forward primer annealing site, the sequence from 27 to 48 nt is shown, and for the reverse primer annealing site, the sequence from 1357 to 1375 nt is shown. The primer sequence is in the grey box. The arrows mark the direction of DNA elongation in the PCR. The *prsE* gene is positioned in the deposited sequence from 79 to 600 nt, and the *prsF* gene is positioned from 676 to 1179 nt.

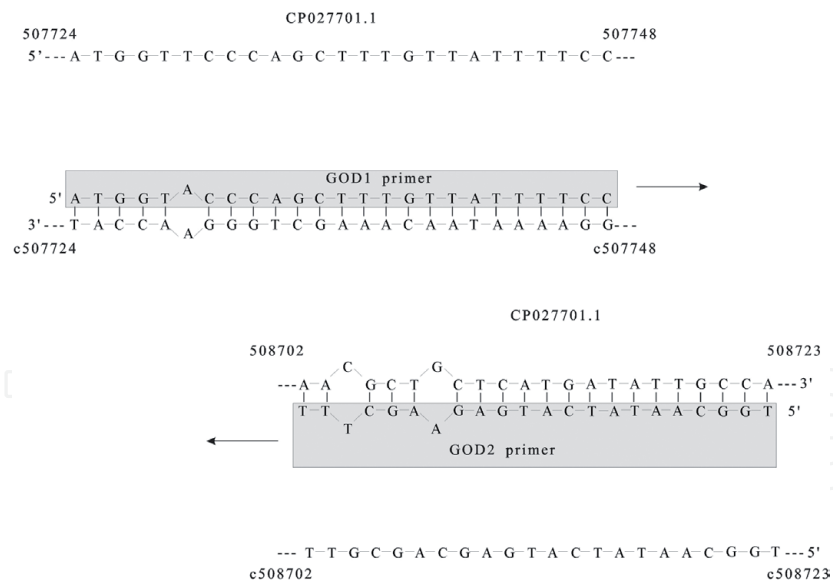


Figure 6. Anticipated annealing sites of primer GOD1 and primer GOD2 in the papG sequence. The shown sequences are enumerated according the CP027701.1 GenBank deposited sequence [37]. The sequence and the complement chromosomal sequence are given. For the forward primer annealing site, the sequence from 507724 to 507748 nt is shown, and for the reverse primer annealing site, the sequence from 508702 to 508723 nt is shown. The primer sequence is in the grey box. The arrows mark the direction of DNA elongation in the PCR. The papG gene is positioned in the deposited sequence from 507716 to 508726 nt.

of GOD1 and GOD2 as revealed by analysis of the nucleotide *E. coli* sequence CP027701.1 [37] are presented in **Figure 6**.

5.4 Analysis of cloned nucleotide sequences from PCRs with primers specific for *F17G*

At the annealing temperature of 55°C with primers specific for the *F17G* gene, eight PCR products, again all of the expected size of approximately 900 bp, were obtained and cloned. Nucleotide sequence analysis of all eight clones showed that

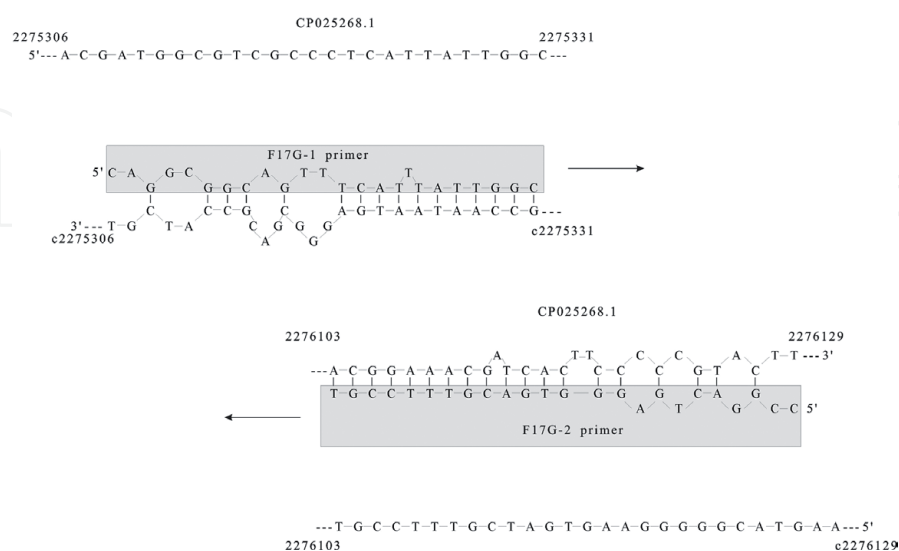


Figure 7. Anticipated annealing sites for non-specific F17G-primer binding in the *rtn* gene. The shown sequences are enumerated according the CP025268.1 GenBank deposited sequence [35]. The sequence and the complement chromosomal sequence are given. For the forward primer, F17G-1, annealing site, the sequence from 2275306 to 2275331 nt is shown, and for the reverse primer, F17G-2, annealing site, the sequence from 2276103 to 2276129 nt is shown. The primer sequence is in the grey box. The arrows mark the direction of DNA elongation in the PCR. The *rtn* gene is positioned in the deposited sequence from 2274762 to 2276318 nt.

four harboured correct and four harboured false inserts. All four false inserts were, as BLAST revealed, sequences of the protein *rtn* gene of the *E. coli* K-12 MG1655 chromosome, as deposited in the CP025268.1 nucleotide sequence [35]. Further nucleotide analysis revealed that in the case of the *rtn* gene amplification, the forward primer F17G-1 annealed downstream from the c2275331 position on the 3' → 5' DNA strand and reverse primer F17G-2 upstream of the 2276103 position on the 5' → 3' DNA strand of the *E. coli* K-12 MG1655 CP025268.1 nucleotide sequence. The anticipated annealing sites for non-specific F17G-primer binding in analysed nucleotide sequences are presented in **Figure 7**.

6. Discussion

The main aim of our research was to determine the sequences of chosen P- and F17-fimbriae genes among *E. coli* isolated from faecal samples of diarrhoeic dogs. As we assumed that the fimbriae of such *E. coli* strains, due to already known variations of P- and F17-fimbriae, might have nucleotide differences, the annealing temperature of 55°C in the PCRs was used. To our surprise, even though only PCR products of expected sizes were cloned, many of the obtained PCR clones, in the case of PCR products obtained with *papA* primers 75% and in the case of PCR product obtained with F17G primers 50%, carried false inserts. Nucleotide sequence analysis revealed that also in the case of *papEF* clones, even though the cloned inserts were as hoped for fimbrial inserts, even if they were Prs-fimbrial genes, the binding site of the reverse primer was not the expected one. The high percentages of false PCR products were obtained when PCR primers with a high melting temperature (T_m) were used at the annealing temperature of 55°C—primer 22 has the T_m of 84.1°C, and 75% of false PCR products were obtained with this primer; primers F17G-1 and F17G-2 have the T_m of 72.4°C and 76.4°C, respectively, and 50% of false PCR products were obtained with them. In the consecutive PCR amplifications with the primers 22 and 23, the annealing temperature was raised to 60°C, and from these PCRs more PCR products were obtained, namely, 16. All 16 were cloned and analysed, and all clones were with correct inserts (data not shown).

7. Conclusion

To conclude, we all know that with PCR, we can obtain false unspecific products, and we believe that such PCR products will be distinguished from right PCR products, because the false PCR products will not be of the correct expected size; however, our results showed that also PCR products of the expected size can be false PCR products. In order to avoid false positive PCR results, it is therefore essential to use the right annealing temperature that should not be too different from the primer's melting temperature.

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Conflict of interest

The author has no conflict of interest.

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