Chapter 8

Analyses of the Polymorphisms in *E. coli* Strains Associated with Heat-Shock Proteins Hsp 55 Isolated from Bird Feathers

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Additional information is available at the end of the chapter

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Abstract

The bird feathers are often colonized by pathogenic microorganisms including mainly bacteria of the *E. coli* species. There is a grooving evidence that due to colonization of the pathogenic bacteria after slaughter material may lead to different zoonosis diseases that endanger human health. Poultry diseases are a very important issue both economically and epidemiologically in each country. Currently, in practice, EU postmortem monitoring programs are often used to eliminate breeding poultry infected with different pathogenic microorganisms, e.g., *E. coli* by introducing mandatory bird vaccination. The article describes the combination of genetic and genomic methods that were used in the analysis of species specificity of strains and their genomes, including specific pathogenic bacteria in bird feathers. The aim of the study was (i) to investigate DNA polymorphisms of the obtained bacterial strains isolated from avian feathers (ii) obtaining recombinant *Hsp55* protein and defining its role as a potential component of vaccines used in poultry diseases. For the detection and analysis of DNA polymorphisms, we have optimized a new innovative method based on the deficiencies of three molecular techniques, AFLP, PCR-MP, and PCR MP. This new method can be a useful tool used in the genotyping of bacterial *E. coli* serotypes present on avian feathers after the slaughter process. It also allows to effectively identify a number of early stages of infectious diseases from heterogeneous avian research material. Amplification of polymorphic regions was achieved by using a lower denaturation temperature of the primers and a reduction in the number of cycles in the classical PCR, which simplifies the procedure, preserving the quality and reproducibility of the obtained results. Research of recombinant *Hsp55* protein has allowed us to determine the optimal conditions for its production by the classical methods used in proteomic analysis.

Keywords: *E. coli* serotypes, bird feathers, DNA polymorphism, ALFP method, LM-PCR method, PCR MP method, Hsp

1. Introduction

Growing urbanization increases the risk of zoonosis diseases that endanger human health. These diseases are caused by microorganisms, parasites, and their toxic biological products. People working in the poultry industry or bird slaughterhouse are exposed by contact with bird feces and dust from feathers of domestic and farm birds. The lack of adequate hygiene and protective clothing can lead to direct skin or respiratory tract infections. Zoonoses actually are infectious transmitted by animals to humans [23, 63]. The people are more susceptible to zoonoses carried than birds, and they share more diseases with them. These infections as etiological factors can develop in humans in several ways: by the respiratory system, digestive tract, and skin laceration [23, 62]. Many microorganisms like bacteria of the *E. coli* strain need proper conditions and parameters to determine their host as receptor on the surface of the cells [23, 62]. These receptors can penetrate into cells, causing the development of infection. The mechanism determines the susceptibility of one animal species to a pathogen and resistance of another species. In the case of people who have been infected with an avian zoonosis, hospital treatment in isolation and prolonged administration of specific antibiotics is required [23, 62]. Avian zoonoses in humans may lead to a chronic disease or death. The first symptoms of a disease in a person appear after 2 weeks, resemble symptoms like food poisoning or influenza, and may also cause meningitis. These diseases are very dangerous for pregnant women or for those with reduced immunity and last for about 3 weeks. A small amount of bacteria introduced into the human body through contact with feathers or contained in feather dust can occur without visible symptoms. The above factors are responsible for the severity of the clinical course of infection, epidemic outbreaks in poultry, increasing antibiotic resistance, and economic losses. So far, the diagnosis of poultry infections has been based on clinical study, and bacteria carriage in bird feathers has not been tested [12].

The main goal of the work is developing the methods for DNA diagnostics toward *E. coli* from bird feathers that shall allow identifying their pathogenic serotypes so that it will be possible to control the environmental factors responsible for epidemic proper treatment in the future.

To better identify the microorganisms, we have decided to combine the three techniques that generate a very large number of polymorphic genomic DNA fragments into one optimization technique which can help significantly shorten the individual steps to obtain a reliable results of the analyzed material. These techniques belong to amplified length fragment polymorphism (AFLP), [1, 37, 39, 57], ligation-mediated PCR (LM-PCR) [17, 41], and PCR MP (polymerase chain reaction melting profiles) described in 2003 by [33] and modified by [24–28]. Currently, in a literature there are no data available concerning of analysis of avian-feathered microorganisms based on genetic polymorphism methods with using one of the available molecular biology techniques. These methods used separately can be helpful in identifying

the kinship between pathogenic strains when the information about the genome is unknown. Therefore, there is a need to clarify this issue.

AFLP as a unique fingerprint is generated for a particular genome and was first developed for plant studies [59]. Although they can generate large numbers of marker fragments for any organism is fraught with some errors. These include potential nonhomology and asymmetry in the probability by loss of fragments. Sometimes, they appear the problems in distinguishing heterozygote from homozygous bands [43, 44, 61]. LM-PCR technique which was discovered and used by [37, 41] for the following modification for footprinting of DMS-induced DNA damage is applied widely by [31]. LM-PCR is a procedure capable of detecting the presence of specially DNA sequences which are determined by a specific protein sequence of enzymes in the phosphodiester backbone of nucleic acid. Also, this technique has problems connected with the proofreading polymerase, which affects significantly the quality received bands [2]. The PCR MP method is based on genomic DNA digestion with only one restriction enzyme, namely, HindIII. This technique could also be used to check the stability of bacterial strains in industrial plants, microbial collections, and epidemiological studies [6, 34, 51]. At present "gold standards" in genomics of microorganisms are methods based on DNA–DNA hybridization, which are quite expensive and difficult to be carried out. But more widespread methods such as AFLP, LM-PCR, or PCR- MP despite their high universality power are less repetitive between laboratories, and their results are difficult to analyze [6, 13].

Based on the deficiencies of these techniques, we have optimized them in new one method in which uses multiple digestion sites to analyze DNA from environmental samples. This method can be a useful tool used to identify phenotypic characteristics of bacterial pathogen *E. coli* present in avian feathers after the slaughter process. This new compiled technique can be in the future a potential marker to distinguish epidemic from endemic strains and chance as a tool used routinely in microbiological diagnostics of bacterial genomes of unknown sequence from different sources as they described [23, 36, 18]. The high repeatability and shorter time of data analysis while preserving the quality of obtaining results facilitate the study of multiple gene loci at a reduced cost [38, 46].

This method is necessary to use DNA of high quality to obtain readable profiles and consists of only three short steps (**Figure 1**).

Briefly, in the first step, a total genomic DNA isolated from a bacterial strain is digested with one or two restriction enzymes giving 5′ or 3′ sticky ends at phosphodiester bond (**Figure 1**). In the second step, double-stranded synthetic short adapters with complementary sticky ends are ligated to the restriction fragments (**Figure 1**). These adapters including an enzyme-specific sequence are formed (leading ends) for complementary sequence of the restriction fragment (hanging ends). The ligated oligonucleotide is a template in PCR. In the third step, PCR amplification with one set of primers complementary to the end-specific target sequences of restriction fragments terminated with an adapter is amplified (**Figure 1**). Only part of the restriction fragments digested by EcoR1 and HindIII is denatured by becoming amplification templates. Next, the amplified DNA fragments are hybridized with the characteristic pairs of universal primers to 16S RNA sequence.

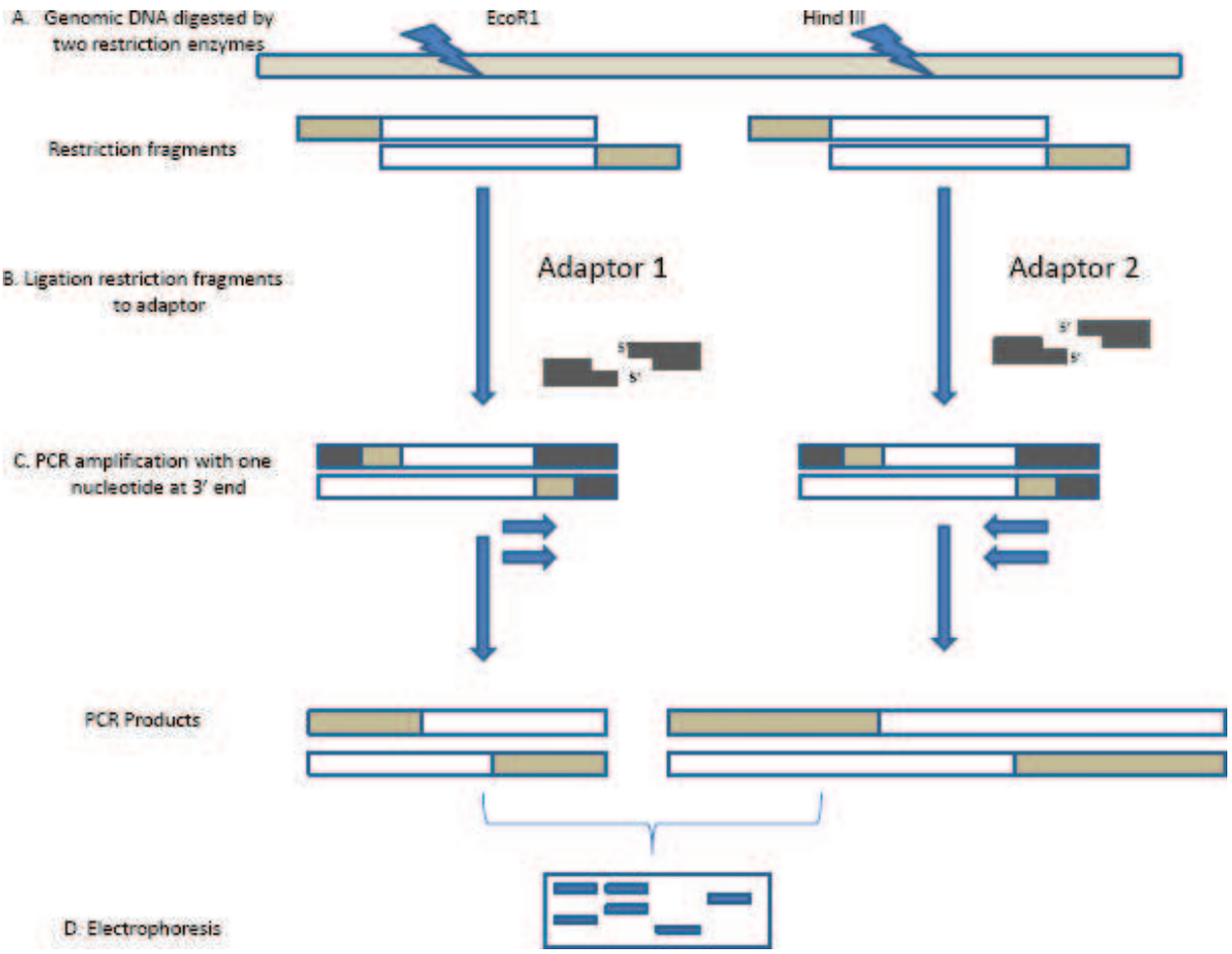

Figure 1. Multiple digestion sites to analyze DNA from environmental samples.

Using standard denaturation temperatures in the range of 90–95°C in PCR, all restriction fragments are terminated with suitable adapters. To obtain a limited amount of amplified fragments (selective amplification), a lower denaturation temperature of up to 90°C in 20 cycles of PCR is used. Due to the different thermal stability of the double-stranded restriction fragments in the analyzed biological material, the selection by lower denaturation temperature increases the distinction potential of analyzed profiles. Next, the products of PCR reaction of PCR, digested by two restriction enzymes of different lengths which are visualized on a polyacrylamide gel electrophoresis a stained with ethidium bromide as part of the analysis of the kinship of individuals analyzed strain. Differences in distances between specific paths in the gel are helpful in unique band pattern interpretation. Parallel with analyzed polymorphisms, we are looking for heat-shock proteins (Hsps) in bacterial cultures isolated from the bird feathers. The presence of these proteins is formed probably in a response to lower reaction temperature in analyzed bacterial cultures. The family of heat-shock proteins (Hsps) belongs to the seven groups of proteins with different molecular masses: 18, 30, 40, 60, 70, 90, and 110 kDa. These proteins are induced in response to cellular abiotic or biotic stress in prokaryotic and eukaryotic cells, mainly induced by elevated temperature. They participate in the stabilization of the cellular components [9].

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The presence of Hsp60 and Hsp70 in bacteria strains can increase their pathogenic functions [16]. Amino acid sequence in Hsps proteins isolated from different microorganisms is very similar and shows high homologues 50% in its construction with eukaryotic proteins [52]. In *Escherichia coli*, Hsps possesses two chaperone systems containing 20% of all proteins at a temperature of 46°C [45], and they perform protective functions in the cell.

2. Materials and methods

2.1. Environmental samples and bacterial strains

In the first stage of the work, the waste and air samples contained 35 various kinds of feathers from poultry slaughterhouses in north-western Poland were used (**Table 1**).

Then, 50 g of each samples from the feathers was added to 450 ml of dilution fluid (0.85% NaCl +0.01% peptone), and the whole was shaken on a mechanical shaker for 2 hours. After this time from the feather samples, bacterial strains by using solid test dilutions were isolated. From this dilution 10^{-1}, further decimal dilutions were made up to (10^{-2} to 10^{-6}). Next, the types of bacterial strains were initially confirmed by:

1. Plate culture: 1 ml of slurry from each subsequent dilution was transferred to two parallel Petri dishes, poured about 15 ml of liquid, and cooled into TBX medium. After thorough mixing and solidification, the culture plates were incubated at 44°C for 18–24 h. The bacterial colony, during the chromogens accumulated in the cells, become blue or blue-green color. Characteristic colonies were isolated, and pure culture was conducted on TSA medium (incubation at 37°C for 24–48 h). Additionally, the bacterial cells were verified by microscope analysis.
2. Procedure with pre-selective multiplication on liquid MacConkey medium (MacConkey Broth). From subsequent decimal dilutions (10^{-2} to 10^{-6}), 1 ml to three parallel tubes containing Durham's tubes was plated. Incubation was carried out at 37°C for 24–48 h. From positive test tubes (yellow color change and gas presence), the reduction media was applied to solidified TBX media. Characteristic colonies based on their morphology were isolated, and pure cultures were grown on TSA and TBX medium (incubation at 37°C for 24–48 h) after 24 h of incubation at 44°C [6]. Additionally, the bacterial species were identified by biochemical testing in the HiBio-ID reader (Himedia Company, India). Preliminary identification of bacterial strains based on biochemical tests showed one type of bacteria, namely, *E. coli* (**Table 2**).

2.2. Isolation of genomic DNA

To confirm the accuracy belonging to the type of analyzed bacterial strains, the genomic DNA were isolated on the large scale by using the AccuPrep Genomic DNA Extraction Kit (Cat. No. K-3032R, Bioneer Company, South Korea) according to the manufacturer protocol (**Figure 2**).

Number of strain	Object
1–12	Chicken feathers
13–18	Turkey feathers
51–56	Chicken feathers
63–67	Turkey feathers
111–115	Duck feathers
116–121	Goose feathers

Table 1. Types of tested bird feathers.

Lp.	Biochemical feature	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	L-Glucose	—	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+	+
2	D-Adonitol	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	—	—
3	D-Arabinose	—	+	+	+	+	—	+	+	+	+	—	+	+	—	+	+	+	+
4	L-Lactose	—	—	—	—	—	—	+	—	+	+	+	+	+	—	+	+	+	+
5	D-Sorbitol	—	—	—	—	—	—	—	+	—	—	+	+	+	+	+	+	+	+
6	D-Mannitol	—	+	—	—	—	+	—	—	—	—	+	—	+	—	+	+	+	+
7	D-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	+
8	D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	+
9	D-Xylose	+	+	+	+	+	+	+	—	+	+	—	+	—	+	+	+	+	+
10	D-Melibiose	—	+	+	+	+	+	—	—	—	—	—	—	+	—	+	+	+	+
11	L-Raffinose	—	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+	+
12	L-Trehalose	—	—	—	—	—	—	+	—	—	—	+	+	+	+	—	—	+	+
		51	54	55	56	63	66	67	111		112	113	114	115	116	117	118	119	121
1	L-Glucose	+	—	—	+	—	—	+	+		+	+	+	—	+	+	+	—	+
2	D-Adonitol	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
3	D-Arabinose	+	—	—	—	+	—	—	+		+	+	+	+	+	+	+	+	+
4	L-Lactose	+	+	+	+	+	+	—	—		+	+	+	—	+	—	+	+	+
5	D-Sorbitol	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
6	D-Mannitol	+	+	+	+	+	+	+	—		+	+	+	+	+	+	+	+	+
7	D-Rhamnose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	—	+
8	D-Sorbitol	+	+	+	+	+	+	+	—		—	+	+	+	+	+	+	+	+
9	D-Xylose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	—	+
10	D-Melibiose	+	+	+	+	—	+	+	—		+	+	+	+	+	+	+	+	+
11	L-Raffinose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
12	L-Trehalose	+	—	—	—	+	+	+	+		+	+	+	+	+	+	+	+	—

Table 2. Example of biochemical characterization of strains isolated from the environmental samples.

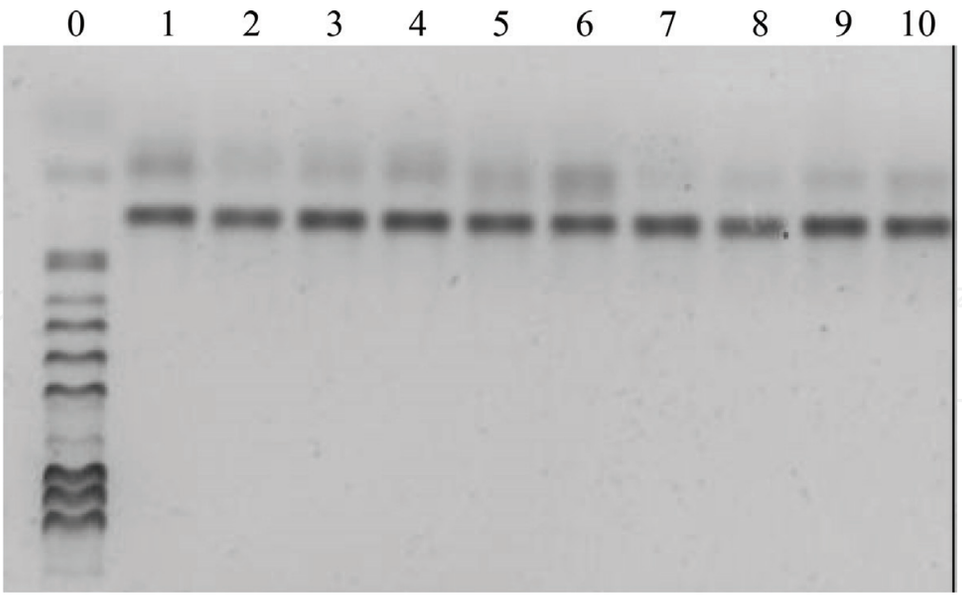

Figure 2. An example of a gel containing genomic DNA isolates. Lane 0, DNA 10 kb marker. Lanes from 1 to 10, DNA genomic samples. Genomic DNA concentration 150–200 ng/μl was determined by UV light absorbance at (A_{260}/A_{280} ratio). The obtained material was either used as a template in a multiplex PCR.

2.3. PCR amplification

To perform PCR amplified from isolated genomic DNA, AccuPower PreMix kits (Cat. No. K-2011, Bioneer Company, South Korea) was used according to the reaction condition recommended by the manufacturer with one pair of universal primers:

ECP79F-forward operating bases. 79–96; 5′-GAAGCTTGCTTCTTTGCT-3′ and ECR620R-(reverse targeting bases 602–620; 5′-GAGCCCGGGGATTTCACAT-3′).

In addition to the tested samples, negative control was performed without DNA, which confirmed the purity of the used reagents. To obtain the same specific product in all amplified DNA samples, optimal primer melting point temperature at 55°C was used. After PCR we received product size at 541 bp which was purified with using AccuPrep PCR Purification Kit (Cat. No. K3034, Bioneer Company, South Korea) as recommended by the manufacturer (**Figure 3**).

2.4. Electrophoresis of PCR products

After PCR, the samples were applied to a 1% agarose gel in 1× TBE buffer (Tris, boric acid, EDTA pH 8.0) containing ethidium bromide in an amount of 2 μl solution at a concentration of 10 mg/ml). The TBE solution was also used as an electrode buffer. In order to estimate molecular length of PCR products, a GeneRuler 1 kb DNA marker (Fermentas) was added to one of the gel pockets. Electrophoresis was performed for 40 min at 90 mV on a Bio-Rad PowerPac Basic. Upon completion of the electrophoresis, the gel was placed on a light-emitting diode (X = 302 nm) and photographed using a GelDoc-It Imaging System and UVP gel visualization system. The stage of electrophoresis informed of the length of the obtained products and confirmed the specificity of the primers used on the basis of the presence of a single gel band. Photos of gels were received and analyzed using Life Science Software Launch VisionWorks (**Figure 3**).

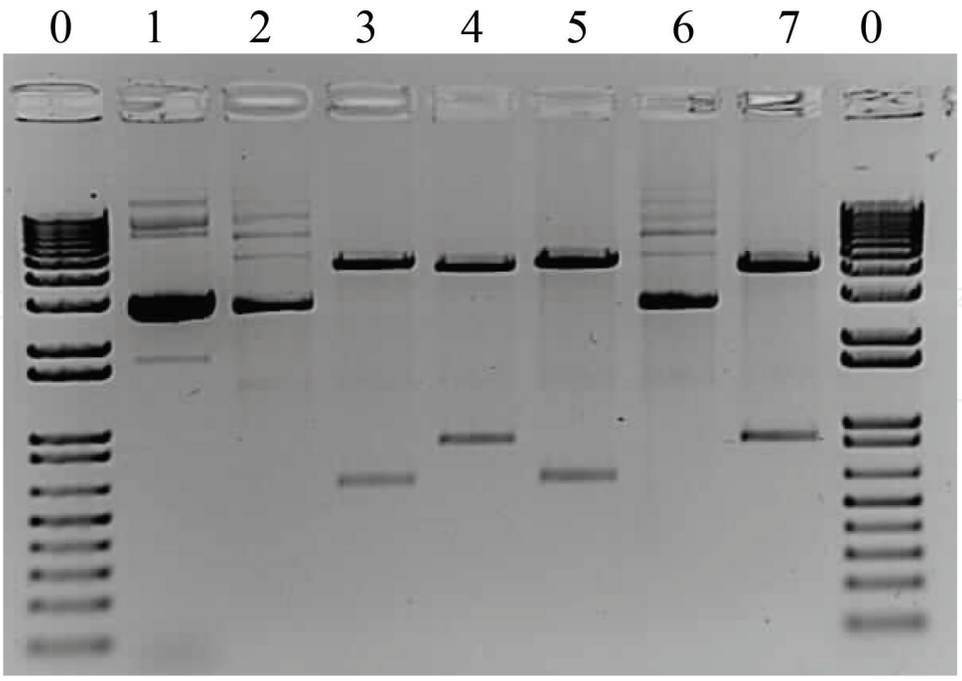

Figure 3. Example of polymorphism fragment in one of the selected strains digested with restriction enzymes EcoR1 and HindIII. Lane 0, DNA ladder 1 kb; lane 1, control DNA digested by both *E. coli* I HindIII enzymes; lanes 2 and 6, digested genomic DNA by both enzymes; lanes 3 and 5, digestion by EcoR1; and lanes 4 and 7, by HindIII.

2.5. Sequencing

Next, the PCR product was sequenced of the 16S rRNA with a universal set of primers; ECB75F (forward targeting bases 75–97; 5′-GGAAGAAGCTTGCTTCTTTGCTG-3′), ECR620R (described above) in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The received nucleotide sequences were compared with sequences located in the GenBank databases/EMBL (European Molecular Biology Laboratory)/DDBJ (DNA Data Bank of Japan) using the BLAST (Basic Local Alignment Search Tool) bioinformatic database. Obtained sequences confirm identification of all *E. coli* strains from ATCC group using biochemical methods including API tests. Among the analyzed sequences in the conserved domains, we have found 10, which had significant differences in the analyzed length of the DNA fragments.

2.6. Digestion by restriction enzymes

On the results of the sequenced DNA, 14 samples for further analysis of polymorphisms were selected. The 20 μg of each sample of genomic DNA samples was digested by combination of two restriction enzymes *EcoR1* and *HindIII* (10 U/μl each) (Fermentas, Lithuania) (**Figure 2**) to get sticky ends. The reaction was run for 2 h at 37°C in a buffer compatible with both enzymes (Tango Buffer: Fermentas, Lithuania) in volume 25 μl consistent with the manufacturer's protocols (**Figure 3**).

2.7. Ligation

After digestion of DNA with restriction enzymes, two pairs of synthetic oligonucleotides forming adaptor were included. First fixed part ligated oligonucleotides are POWIE 5′-CTCACT-CTCACCAACAACGTCGAC-3′ and HINLIG 5′-AGCTGTCGACGTTGG-3′ described by

Masny and Płócienniczak [33] and second fixed part POWIE 5′-CTCACTCTCACCAA-CAACGTCGAC-3′ and EcoR1LIG 5′-AATTGTCGACGTTGG-3′. Both adaptors (25 pmol each) were suspended in total volume 25 μl of ligation buffer (100 mM Tris–HCl, pH 8.2, 10 mM $MgCl_2$, 15 mM DTT, and 100 mM ATP; Bioneer South Korea). Next, the adaptors were ligated to the "sticky ends" created by both restriction enzymes. The reaction was incubated either for 5 min at 55°C and gradually cooled to room temperature. Then, 2 μl of ligase T4 2 U/μl (Cat No. E-3061, Bioneer, South Korea) was added, and reaction mixtures were incubated during 2–4 h at room temperature according to manufacturer's protocol. As a control, a sample of water was placed in place of the DNA fragment. Obtained construct in volume 5 μl was used for transformation of the competent cells of DH5alpha BL21 (Stratagene) as described by Sambrook and Maniatis [44]. Next, the transformation mixture was plated on LB medium with ampicillin (50μg/1 ml) and incubated at 37°C overnight. After incubation obtained cells including construct were frozen in Cryobank (Kucharczyk, Poland) and were stored for further molecular analysis at −80°C.

2.8. Colonial PCR

Cells including construct after defrosting were incubated at 37°C for 10 min and served as matrices to the colonial PCR. The initial primer concentration was 100 μM. The PCR mixture contained 60 pmol of primer POWAGCTT 5′-CTCACTCTCACCAACGTCGACAGCTT-3′ described by Masny and Płócienniczak [33]. To amplify the Hsp55 protein gene fragment, the PCR mixture in 25 μl volume composition was consistent with the manufacturer's protocols (*AccuPower® Pfu* PCR PreMix Cat No. K2024 Bioneer Company, South Korea). PCR program was as follows: (1) 90°C 4′, (2) 56°C 2′40″, (3) 72°C 3′40″, (4) goto1 1×, (5) 90°C 1′40″, (6) 52°C 2′40″, (7) 72°C 3′40″, (8) goto5 20×, (9) 72°C 7′40″, and (10) 4°C forever. The reactions were performed in Bio-Rad MJ Mini thermocycler. After completion of the PCR, the samples were transferred quickly into ice before loading on the 6% polyacrylamide gel.

2.9. Polyacrylamide gel electrophoresis

Next, the amplification PCR products (6 μl out of 50 μl DNA solution) were separated on 6% polyacrylamide gel electrophoresis in conditions 90 min under 120 V in the presence of TAE buffer (Cat. No. C-9004, Bioneer, South Korea) supplemented in 0.5 mg/L aqueous solution of ethidium bromide (Cat. No. C-9009 Bioneer, South Korea). All samples were runs the ladder Gen Ruller were runs the ladder GeneRuler 1000 bp (Cat. No. D-1040, Bioneer, South Korea) (**Figure 4**). The differences between digested templates were analyzed using ImageQuant software.

2.10. Production of recombinant *Escherichia coli* Hsp55 protein

Additionally, we also wanted to check whether the analyzed bacterium pellets, based on differences in DNA polymorphisms in 10 samples, are able to detect specific heat-shock proteins related to them. Based on genomic DNA obtained from bird feathers and digested with restriction enzymes as insert (as described in Section 2.6), we used a vector pET22b (5493 bp) (Novagen, Germany). A vector is based on the T7 promoter system, and its expression is controlled by the lactose operon. Additionally, to digestion the vector of the vector pET22b on sticky ends, the same types and portion of restriction enzymes EcoR1/HindIII were used on

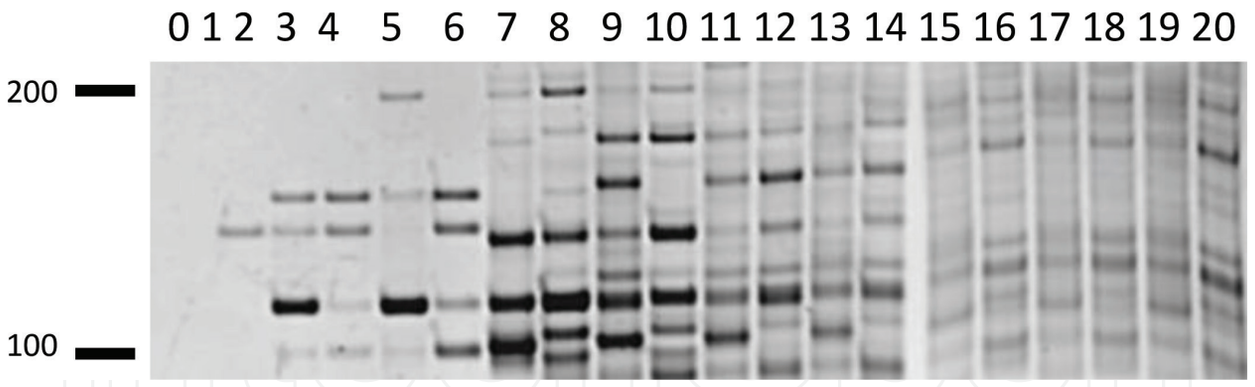

Figure 4. Analyses the differences between DNA templates isolated from *E. coli* strains with using the modified technique on polyacrylamide gels stained with ethidium bromide. Lane 0, molecular weight standard 2 kb (Bioneer). The remaining lanes 1–20 show digested profiles by both restriction enzymes: even numbers by EcoR1 and odd numbers by HindIII.

sticky ends (as described in Section 2.6). Next, the insert and vector were ligated with themselves by ligase 2 μl of ligase T4 2 U/μl in the same conditions (as described in Section 2.7). The obtained construct called hsp55pET2'b was transformed into competent cells of *E. coli* BL21, (Stratagene) (as described in Section 2.7). Next, the bacterial cells were used to express the hsp in autoinduction conditions according to Studier [50] (see Chapter 2.11).

2.11. Autoinduction

After overnight culture at 37°C, the bacteria were centrifuged for 20 min at 2500 × g, the supernatant was flushed, and the bacterial pellets were purified by affinity chromatography with a histidine oligopeptide (His-Tag) (see Chapter 2.12) under denaturing conditions (application of 4 M urea) on Sepharose Ni_2 gel. Next, the recombinant Hsp55 *E. coli* proteins obtained in the previous step were dialyzed in Tris-NaCl buffer. The dialysis buffer was changed five times. After dialysis, protein concentrations at (λ = 280) were determined.

2.12. Affinity chromatography

A chromatographic column Ni-NTA (Sigma-Aldrich, Germany) with a nickel agarose gel was used to purify the protein. The column has the properties of attaching molecules to the polyhistidine tail (His-Tag tail attachment). The protein was purified according to the Invitrogen protocol (Ni-NTA Purification System, Sigma-Aldrich, Germany). Quantitative evaluation of the protein was carried out on a regular basis using 100 μl Bradford Reagent (Bio-Rad). The concentration of proteins measured spectrophotometrically was ranged from 0.35 to 0.36 μg/ml.

2.13. Protein electrophoresis

For the separation of protein fractions obtained by affinity chromatography, SDS-PAGE was performed at 60 mA (**Figure 5a**). The gel composition was according to the manufacturer's protocols (Bio-Rad). The composition 20 μl of sample, 5 μl of dye, and additionally 7 μl of PageRuler™ Unstained Protein Ladder (Lab-JOT, New England, Great Britain) were applied

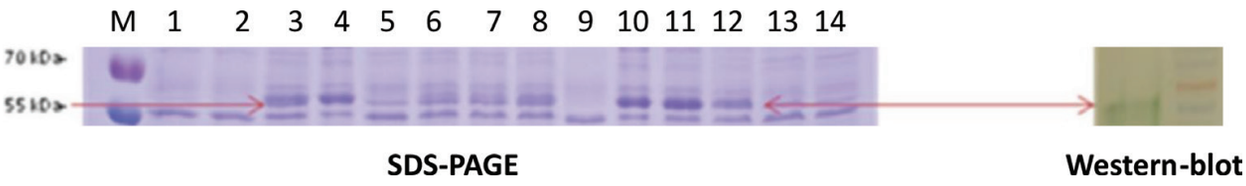

Figure 5. Analysis of the expressed proteins by SDS-PAGE (A) and Western blot (B). SDS-PAGE protein profiles of the *E. coli* strain stained with Coomassie Brilliant Blue. Lanes: M, molecular mass standard 6.5–200 kDa; 1–14 analyzed protein fractions. Red arrows show protein fractions of molecular weight 55 kDa.

to two gel lanes. The bands migrate at a height of 55 kDa relative to the protein ladder, and the absence of additional intermediate bands at this height of the template protein indicates a high degree of purification of the analyzed proteins. After electrophoresis the gel was stained with Coomassie Brilliant Blue (Sigma-Aldrich, Germany) according to the manufacturer's protocols. Next, the first gel was transferred on nitrocellulose membrane (Western blot) to confirm the acquisition of protein *Hsp*55 (**Figure 5b**), (see Chapter 2.14).

2.14. Western blot

Transfer from polyacrylamide gel to nitrocellulose was carried out by semidry method [53]. The gel after the electrophoresis was transferred to a soaked transfer buffer (30 mM Tris, 180 mM glycine, 25% methanol, pH 8.2) Whatman paper (Sigma-Aldrich, Germany) according to the scheme: upper transfer (−) 3 Whatman paper 4, 1 Whatman paper 1, gel, nitrocellulose membrane, 1 Whatman paper 1, and 3 Whatman 4 paper (+) down. The transfer was at a current of 1.8–2 mA/cm^2 of the membrane for 45 min. At the end of the transfer, nitrocellulose was blocked with a solution of PBS + 2% casein for 15 min. On the membrane goat anti-mouse primary antibody (IgG anti-His-Tag 1:2000, Sigma-Aldrich) were applied and were incubated overnight at 4°C. After incubation, the membrane was washed three times with PBS + 0.025% Tween 20, followed by a 1:2000 conjugate (Anti-Goat, Santa-Cruz, USA) conjugated with HRP and incubated for 1 h at room temperature. The next step was to rinse the membrane three times with PBS + 0.025% Tween 20 solution and to induce nitrocellulose (20 mg chloronaphthol dissolved in a minimum of 96% ethyl alcohol), added to 80 ml Tris–HCl buffer and then filtered through a paper filter. Protein samples after chromatography were conserved in 20% glycerol solution and frozen at −20°C. The resulting membrane, as well as the SDS-PAGE gel, was scanned and analyzed.

3. Results and discussions

The traditional polymorphism technique is a compilation of digestion by restriction enzymes and selective basic PCR, which can generate a wide spectrum of markers [24]. These markers are highly specific and needs typical sequence of the data base. Additionally, they require visualization platforms which can be intractable to analysis by specific banding patterns.

Our studies show that modification of these presented techniques can be an effective alternative to other PCR methods used to identify specific DNA sequence occurring in environmental origin from bacterial pathogens, e.g., bird features. Using these modified techniques, we observed that all 10 samples isolated from the same bird feathers treated by restriction enzymes presented different profiles. The reliability and repeatability of modified methods depend on optimizing the PCR conditions [24, 33] by experimental estimation: the amount of DNA of only 0.2 μg (0.2 pmol) of DNA per reaction is needed to obtain satisfactory signal intensity, the amount and concentration of reagents (what affects it reduced cost), the primer denaturation temperature 90°C, or the number of temperature cycles—only ×20, which significantly shortens the time of the experiment. We describe a method of finding differences in DNA sequence among bacterial species strains based on two enzymes EcoR1 and HindIII [24, 33, 61]. The method allows on analysis of DNA fragments, whose band pattern on the gel differs between strains of a bacterial species. The DNA sequences obtained from bird feathers are colonized by the serogroups of bacteria belonging to *E. coli* species (**Table 3**) [11, 23]. The most commonly isolated avian pathogenic *E. coli* strains were O6, O7, O26, O55, O127, and O157 which are the main causes of colicobacteriosis, inflammation of air sacs, polyserositis, sand bacteremia and epidermis [35]. Probably, the factor predisposing to the development of these infections is the PICV (circovirus) infection, which affects molting disorders, leading to immunosuppression and development of parenteral infections like lymphatic tissue in the fabrician bursa, thymus, spleen, and intestinal tract [3, 42, 23]. The most important virulence factors of *E. coli* colonizing bird feathers are fimbriae F1 and P, aerobactin inducer iron-sequestering system (AIS), and temperature-sensitive hemagglutinin [21]. They promote the development of generalized infections and inhibit phagocytosis in poultry lung alveoli in case of *E. coli* aspiration [49]. The obtained strains IAI 39 and 536 with serotypes O7:K1 and O6:K15:H31, respectively, are the etiological agents of pyelonephritis (ExPEC). Other strains EDL933 (O157:H7 serotype), CB9615 (O55:H7 serotype), APEC01 (01 serotype), and E2348/69 (O127:H6 serotype)

***E. coli* strain name**	**Serotype**	**Pathotype**	**Complete genome length G + C ratio [%]**
SS17	O157:H7	HUS	5.52 Mb/50.5%
SS52	O157*nonH7*(H16, H26, H39)	pEAF	5.488 Mb/50.5%
CB9615	O55:H7	Diarrhea	5.52 Mb/50.5%
E2348/69	O127:H6	Diarrhea	5.52 Mb/50.5%
APEC01	O157:H7	Diarrhea	5.498 Mb/50.5%
EDL933	O157: K1:H7	Diarrhea	5.498 Mb/50.5%
536	O6:K15:H31	Diarrhea	5.528 Mb/50.5%
39	O7:K1	Diarrhea	5.528 Mb/50.5%
EC4115	O157:H7	EHEC	5.57 Mb/50.5%
TW14359	O26:H11	HUS	5.528 Mb/50.5%

Table 3. Summary presentation obtained *E. coli* pathogenic strains including serotype.

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are responsible for causing diarrhea, with symptoms of colicobacteriosis and secondarily of inflammation of air sacs, polyserositis sand bacteremia and epidermis (**Table 3**). Among the carriers of *E. coli* strains producing O157 (EC4115, EDL933, TW14359, SS17, SS52), we can distinguish the group determined as super-shedders—contamination O157 ≥ 10^4 CFU/g [7, 14, 23]. In addition, due to genomic similarity in the sequence length (5.49–5.53 Mb), G + C (50.4%–50.5%) proportion, and the average ORF (856–898 bp) length of *E. coli* strains, the reliable methods of genetic diagnostics that will allow to identify the pathogenic strains definitely are sought for (**Table 3**) [13, 23], which is consistent with the results obtained in other laboratories, which are described in the review paper [23].

Based on the analysis of the length of the restriction fragment, we can state that each analyzed lane is from 4 to 35 DNA fragments (bands) of approximately range from 150 to 2072 bp. Profiles found were different in relation to the presence or absence of at least one band, and all of them presented one band of approximately 1500 pb. The total number of the bands was 432, clearly separated and good visualized on gels only. Among primer combinations (see chapter primer selection 3.2), producing 14 polymorphic fragments, respectively, [57] reported that fragments amplified by modified method can detect from 60 to 100 fragments on a polyacrylamide gel. Genetic polymorphism, understood as the presence of more than one allele at a given locus in the population, can be identified by genetic markers [10, 57], using molecular methods based primarily on AFLP, LM-PCR, and PCR MP. In our modified methods, we can detect two times more fragments for revealing polymorphism. A high resolution of obtained gel stained in ethidium bromide revealed to be an efficient technique to visualize markers in modified method [33]. Samples belonging to the same biotype when tested by modified method presented small differences of the patterns. The differences between the two classes of represented isolates suggest probably a clonal relationship between the strains. It is well known that the presence of transposons, different types of mutation in sequences, may increase the variability of the profiles produced by the modified- three techniques. Modified technique may be useful for studying intraspecific variation and genetic relationships among different biotypes. According to the up-to-date reports, conventional PCRs or real-time PCRs are useful in detecting different pathogen strains [24]. DNA sequences that are characteristic of certain strains of a bacterial species can be very interesting from the biological and medical point of view. Bacterial genomes show considerable variation in GC content. This means that after genomic DNA digestion, fragments with different thermal stabilities are formed. The resulting sets of electrophoretic patterns are characteristic of the genome and restriction enzyme.

Nonetheless, they may serve as markers for species/strain identification or evolutionary studies. The classical genetic marker is characterized by the identified degree of variability of a feature (DNA nucleotide sequence) and may involve variation within encoding or noncoding sequences or single-nucleotide changes in DNA fragments. The sources of variation in DNA sequences are mutations, among others insertion, deletion, substitution, and duplication, and recombination during meiosis. Some of these changes at the genome level can lead to the variability observed at the phenotypic level, significant from an evolutionary point of view [57]. With respect to natural selection, markers may be neutral or not. It is assumed that neutral markers are not selectable, i.e., specific genotypes or alleles at a given locus or loci are not favored by selection. One of the most commonly used neutral markers are microsatellite sequences, which are used in population structure analysis. Microsatellite locus is a

DNA sequence consisting of short, one to six nucleotides, repetitive elements in the number of different copies for individual alleles. A variety of PCR-based methods for displaying DNA sequence polymorphism have been developed [24, 33]. Compilation of these methods allows detection of DNA polymorphisms distinguishing between strains of a species but does not exhibit high rates of reproducibility. Modified methods permit selective amplification of restriction fragments obtained from a total restriction enzyme digestion of a DNA and identification of DNA polymorphism within a species but require the use of sequencing gels and usually labeled primers because of the quantity of simultaneously amplified DNA fragments.

Our method allows simple identification of microsatellite loci by identifying flanking regions in individuals of the same species. Based on the experimental results, we can observe changes in modified patterns in 10 isolates of the environmental samples (avian feathers), which suggests that the technique is highly specific and presents high variability. The method can also be used for a further variety of epidemiological studies as a tool for the detection of cross-contamination, determination of the source of infection, and recognition of outbreaks and virulent strains [4, 15, 22, 23, 54]. One of the most common causes of bacterial infections in humans is *Escherichia coli*.

Pathogenesis and sources of *Escherichia coli* infection are described by [23, 54]. In poultry as a result of *E. coli* infection, there is a great economic loss, so monitoring programs have been introduced to limit them. Understanding the mechanism of pathogenesis of *Escherichia* species, including the penetration of rods into the intestinal epithelium, has contributed to many experimental studies. Elimination of cases of morbidity of *Escherichia* of morbidity of *Escherichia* has become a common poultry vaccination against avian influenza caused by [23, 54]. Initially used vaccines were based on full bacterial cells (live strains). This leads to numerous side effects, allergies, or toxic symptoms and to the conversion of the vaccine strain to the wild strain. With the development of the study, the attention of the investigators was reversed on bacterial cell fragments that contained a specific antigen [23]. Examples of such antigens may be proteins in the cell membrane of bacteria and intracellular proteins such as heat-shock proteins (HSP). Heat-shock proteins (Hsps) are stress proteins, which are characteristic of prokaryotic and eukaryotic cells. They are rapidly synthesized in response to shock induced by exposure on internal and external factors, e.g., heavy metal ions, high or low temperature, gamma and beta radiation, or various infectious agents. They play a role as stabilizer of cellular structures. In bacteria are documented Hsp60 and Hsp70 documented are Hsp60 and Hsp70 [16, 52]. So far has been identified six protein families with molecular masses between 18 and 30 kDa, 40, 60, 70, 90, and 110 in *Escherichia coli* have been identified, but so far there are no direct data about hsp55 protein. It is the heat-shock proteins that pay particular attention not only to the great conservatism of the construction but also to their high immunogenicity. The ability to use heat-shock proteins in the immune response and their potentially protective role in relation to Gram-negative infections has been the inspiration for the research. The method of production of recombinant Hsp60 protein based on literature data was used [52]. In addition, the results of optimization studies on the production of recombinant Hsps have been confirmed by [52]. The pET21b vector was used in the cloning process, and the resulting construct was cloned into competent cells of *E. coli* BL21 or DH5α.

These are universal competent cells in which effective expression of both membrane and intracellular proteins occurs. In addition, these cells are highly effective in autoinduction, as described by [50]. As a result of the optimization of the production conditions of the recombinant protein, the efficiency of the method used gave 0.35 μg. Available data from [47] present a recombinant protein production process using the Hsp60 protein derived from *Bacillus anthracis* (strain 34F2). For the amplification of the gene responsible for encoding the Hsp55 protein, suitable primers were used, and as a result, a 1635 bp product was obtained which was subjected to restriction analysis with EcoR1/HIII enzymes. The vector used in the cloning process was vector pET28a. The obtained construct was cloned into competent cells of *E. coli* DH5α. The expression process of the protein was based on the classical method using IPTG (synthetic lactose). The protein was purified with Ni-NTA in native conditions and then dialyzed in PBS with 10% glycerol added. The interaction between the *E. coli* pathogen and the host defense mechanism is stimulated probably by the immune system. The primary response to mobilizing the first line of defense (including macrophages) is to eliminate *E. coli* from the body, as demonstrated by [19]. Heat-shock protein 55 found in analyzed bird feathers may belong to the family of hsp present in prokaryotic cells such as *Helicobacter pylori* and *Mycobacterium leprae* [30, 47]. One of major heat-shock proteins in *H. pylori* is HSP60 [8, 58]. The HSP60 protein is expressed on the surface of bacterial cell using the adhesion process during exposition to elevated temperatures [8, 58]. HSP has several chaperones which can activate and regulate other proteins [60]. Some chaperones contain proteins HSP70 and HSP90 [60] which are helpful in supporting the establishment of protein complexes and prevention of unwanted protein aggregation [5, 56]. Finding the Hsp55 protein in various bacterial cultures from analyzed fractions may depend on many abiotic or biotic factors which may influence on the pathogenicity of bacteria. Among all analyzed samples, only eight samples had a clear band at a marker height of 55 kDa. The presence of Hsp55 protein observed only in lanes 3, 4, 6, 7, 8, 10, 11, and 12 of SDS-PAGE gel may indicate that this protein has a multidomain structure, as described by [32, 48]. Obtained results could suggest that the presence of these proteins can be an element of the bacterial cell adaptation to worse thermal condition factors of the environment. The overexpression of Hsp55 protein observed in selective fractions may indicate probably cross-reactions occurring in a homology between Hsp60 and another low-molecular-mass proteins or may be the result of degradation of proteins possessing higher molecular mass than 60 kDa in another microorganisms as described by [29, 55]. The strongest expression of Hsps occurred in the SDS-PAGE gel may suggest that the bacterial fraction was a target site for these proteins. Similarly, the strongest effect was observed in Western blot in selected bacterial cells with anti-Hsp55 antibodies. The positive reactions with Hsp55 protein may indicate a high degree of specificity in the *E. coli* proteins. Probably, in analyzed pathogenic bacterial cultures, stress factors which are crucial for expression of hsp are specific. Perhaps, the stress factors can change the localization of proteins in cells induced with specific gradient which indicates their differences in conformation and physical properties. This may lead to destabilization intracellular structures under stress conditions [20, 40]. The changes of temperature induced Hsps in bacteria, and it can also be a stress factor increasing the expression Hsps in *E. coli* bacteria. In these processes are genes activated responsible for encoding these proteins. Western blot shows that it is a suitable method of identifying this type of protein in analyzed bacterial strains.

To date, the effect of the Hsp55 protein on the immune cells has not yet been fully described. Much attention is devoted to research aimed at learning the response of immune system cells following infections with *E. coli*.

3.1. Selection of primers used in the modified method

One of the most important factors determining the success with modified ALFP and LM-PCR and PCR MP is the design of a gene-specific primer set. Three sets of specific primers must be used for analysis of bacterial strains to check the mapping of the modified method.

Primer set 1 is used for formation of adaptors and their complementary sequence described by [33], primer set 2 is used to make a hybridization probe, and primer set 3 is used for the PCR step. The three sets of primers must be in close proximity or preferably slightly overlapping to avoid repetitive DNA sequences in the bacterial genome and to avoid polymorphic sites. The initial criterion used to test the relationship between bacterial strains was the hybridization of total DNA supplemented with electrophoresis karyotypes and used profiles of restriction enzymes. The most distinctly different fragments were selected from the 6% polyacrylamide gel after silver staining with separated amplification products of individual bacterial strains depicted in the (**Figure 3**). Analysis of isolated strains started from the design of specific primers to obtain the same specific product in all genomic DNA amplified samples by PCR method. PCR primers have been designed to be longer than ligated oligonucleotide's normal primers [33]. Fragments that arise after genomic DNA digestion differ in the melting point and can be amplified directly in PCR after denaturation in 90°C. Using reduced denaturation temperatures, a smaller number of DNA fragments is obtained than at the standard temperature of 95°C, where all the fragments would be amplified. In procedure that uses Taq polymerase for primer extension, primer 1 must have a melting temperature considerably higher than 48°C to remain hybridized at this temperature. Primer 2 should have a melting temperature higher than primer 1 but similar to the linker primer in the PCR step, as they must work as a pair. Thus, for the standard protocol and linker, one needs to select primer 2 with a T_m of 55–59°C. The oligonucleotide used as primer 1 has AT-rich regions; longer primers may be needed to give the desired T_m. For many experiments involving comparisons between lanes, it is desirable to have roughly the same band intensity in each lane. The strain-specific primer used in the amplification step (primer 3) was designed to be downstream to primer 1, overlapping several bases with primer 1 to provide competition with any residual primer 1 that might be carried with the sample to the PCR step. The linker primer was the longer oligonucleotide (the 23–26 mer, T_m of 53.4°C) with a T_m very similar to that of primer 2. Only 20 cycles were performed, so as to ensure enzyme excess and minimize preferential amplification of short sequences. To completely extend all DNA fragments and uniformly add an extra nucleotide by the terminal transferase activity of Tag polymerase, an additional longer elongation step is part of standard procedure. This step can be important and should not be omitted; otherwise, double bands may appear due to some molecules having an extra 3′ base.

4. Conclusions

In conclusion, for the first time, we showed the developed modified AFLP, LM-PCR, and PCR MP methods as one powerful technique for the specific and selective detection of bacterial pathogen from environmental samples, for example, bird feathers. This new method is an

alternative for another research techniques based on PCR. It is more sensitive, much faster, and less labor intensive than the other existing methods. The advantages of this method can be applied to analyze specific fingerprints of obtained DNA (as PCR product) from different bacterial strains after specific restriction enzyme treatment and interaction between the recombinant Hsp55 protein and the humoral and cellular response of the breeding bird organisms. Compilation of these methods is a very useful and flexible tool in the analysis of microbiological strains. It allows analysis of many genomes coming from different sources in a short period of time in lower PCR temperature with small amount of DNA which is an alternative to differentiate between highly clonal strains. Typically, 60–100 restriction fragments are amplified in range size from 60 to 550 bp. The selection of the denaturation temperature is a very important factor for reliability of typing for each strain from previously purified bacterial colony.

The results of the Hsp55 *E. coli* protein evaluation study in birds from breeding farms indicate that it may be used in the future for the construction of component vaccines used in the pharmaceutical industry against various infections responsible for bird conditions. The results presented in this paper demonstrate the interaction between the recombinant *Hsp55* protein and the humoral and cellular response of the breeding bird organisms.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors made substantial contributions to conception and design, analysis, and interpretation of data. In particular, PK designed this project, and PK, SMO, KC, AM, and AM1 also contributed to the laboratory analysis. PK is involved in drafting the manuscript. SMO and KC also participated in sampling and bacterial strain collection operations. PK, KC, SMO, AM, and AM1 reviewed the manuscript for intellectual content. All the authors have read and approved the final manuscript.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement

All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Abbreviations

HUS	hemolytic-uremic syndrome
pEAF	adherence factor plasmid

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References

[1] Althoff DM, Gitzendanner MA, Segraves KA. The utility of amplified fragment length polymorphisms in phylogenetics: A comparison of homology within and between genomes. Systematic Biology. 2007;**56**:477-484

[2] Bogush ML, Velikodvorskaya TV, Lebedev YB, Nikolaev LG, Lukyanov SA, Fradkov AF, Pliyev AF, Boichenko MN, et al. Identification and localization of differences between *Escherichia coli* and *Salmonella typhimurium* genomes by suppressive subtractive hybridization. Molecular & General Genetics. 1999;**262**:721-729

[3] Bougiouklis PA. Avian circoviruses of the genus Circovirus: A potential trigger in Pigeon breeder's lung (PBL)/Bird fancier's lung (BFL). Medical Hypotheses. 2007;**68**:320-323

[4] Bose T, Venkatesh KV, Mande SS. Computational analysis of host-pathogen protein interactions between humans and different strains of enterohemorrhagic *Escherichia coli*. Frontiers in Cellular and Infection Microbiology. 2017;**7**:128

[5] Borges JC, Ramos CH. Protein folding assisted by chaperones. Protein and Peptide Letters. 2005;**12**(3):257-261

[6] Chalhoulb BA, Thibault S, Laucou V, Rameau C, Höfte H, Cousin R. Silver staining and recovery of AFLP amplification products on large denaturing polyacrylamide gels. BioTechniques. 1997;**22**:216-220

[7] Delisle G, Ley A. Rapid detection of *E. coli* in urine samples by a new chromogenic β-glucuronidase assay. Journal of Clinical Microbiology. 1989;**27**:778-779

[8] De Maio A (1999) Heat shock proteins: Facts, thoughts, and dreams. Shock 11(1):1-12

[9] Del Re B, Bersani F, Mesirca P, Giorgi G. Synthesis of DnaK and GroEL in *Escherichia coli* cells exposed to different magnetic field signals. Bioelectrochemistry. 2006;**69**:99-103

[10] Despré L, Giells L, Redoutet B, Taberlet P. Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Molecular Phylogenetics and Evolution. 2003;**27**:185-196

[11] Dho-Moulin M, Fairbrother JM. Avian pathogenic *Escherichia coli* (APEC). Veterinary Research. 1999;**30**:299-316

[12] Dijkman R, Feberwee A, Landman WJ. Validation of a previously developed quantitative polymerase chain reaction for the detection and quantification of *Mycoplasma synoviae* in chicken joint specimens. Avian Pathology. 2013;**42**:100-107

[13] Eaton AD, Clesceri LS, Greenberg AW, editors. Standard Methods for the Examination of Water and Wastewater. 21st ed. Washington: APHA; 2005

[14] Ferens WA, Hovde CJ. *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. Foodborne Pathogens and Disease. 2011;**8**:465-487

[15] Ferdous M, al e. Virulence, antimicrobial resistance properties and phylogenetic background of non-H7 enteropathogenic *Escherichia coli* O157. Frontiers in Microbiology. 2016;**7**:1540

[16] Fleshner M, Johnson JD. Endogenous extra-cellular heat shock protein 72: Releasing signal(s) and function. International Journal of Hyperthermia. 2005;**21**:457-471

[17] Reisig F, Kremer K, Amthor B, van Soolingen D, Haas WH. Fast ligation-mediated PCR, a fast and reliable method for IS*6110*-based typing of *Mycobacterium tuberculosis* complex. Journal of Clinical Microbiology. 2005;**43**(11):5622-5627

[18] Fraga AP, de Vargas T, Ikuta N, Fonseca AS, Celmer AJ, Marques EK, Lunge VR. A multiplex real-time PCR for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in clinical samples from Brazilian commercial poultry flocks. Brazilian Journal of Microbiology. 2013;**44**:505-510

[19] Groesdonk HV, Schlottmann S, Richter F, Georgieff M, Senftleben U. *Escherichia coli* prevents phagocytosis-induced death of macrophages via classical NF-κB signaling, a link to T-cell activation. Infection and Immunity. 2006;**74**(10):5989-6000

[20] Guyot S, Pottier L, Ferret E, Gal L, Gervais P. Physiological responses of *Escherichia coli* exposed to different heat-stress kinetics. Archives of Microbiology. 2010;**192**:651-661

[21] Kobayashi RK, Gaziri LC, Vidotto MC. Functional activities of the Tsh protein from avian pathogenic *Escherichia coli* (APEC) strains. Journal of Veterinary Science. 2010;**11**:315-319

[22] Kowalczyk P, Mahdi-Oraibi S, Misiewicz A, Gabzdyl N, Miskiewicz A, Szparecki G. Feather-degrading bacteria: Their biochemical and genetic characteristics. Arabian Journal for Science and Engineering. 2017. DOI: 10.1007/s13369-017-2700-2

[23] Miskiewicz A, Kowalczyk P, Oraibi SA, Cybulska K, Misiewicz A. Bird feathers as potential sources of pathogenic microorganisms: A new look at old diseases. Antonie van Leeuwenhoek, Journal of Microbiology. 2018;**111**(3):1-15. ISSN 0003-6072. DOI: 10.1007/s10482-018-1048-2

[24] Krawczyk B, Samet A, Leibner J, Śledzińska A, Kur J. Evaluation of a PCR milling technique (PCR MP) technique for bacteria strain differentiation. Journal of Clinical Microbiology. 2006;**44**:2327-2332

[25] Krawczyk B, Leibner J, Stojowska K, Bronk M, Samet A, Kur J. PCR melting profile method for genotyping of vancomycin-resistant *Enterococcus faecium* isolates from hematological unit patients. Journal of Microbiology. 2007;**56**:65-70

[26] Krawczyk B, Leibner J, Samet A, Kur J. PCR melting profile technology—Possibilities for use in epidemiological studies. Medycyna Doświadczalna i Mikrobiologia. 2007;**59**:5-16

[27] Krawczyk B. Molecular diagnosis in hospital infections. Post Microbiol. 2007;**46**:367-378

[28] Krawczyk B, Kur J, Stojowska-Swędrzyńska K, Śpibida M. Principles and applications of ligation mediated PCR methods for DNA-based typing of microbial organisms. Acta Biochimica Polonica. 2016;**3**(1):39-52

[29] Lemos JA, Burne RA, Castro AC. Molecular cloning, purification and immunological responses of recombinants GroEL and DnaK from *Streptococcus pyogenes*. FEMS Immunology and Medical Microbiology. 2000;**28**:121-128

[30] Liao KW et al. Antibodies against *Helicobacter pylori* heat shock protein 60 aggravate HSP60-mediated proinflammatory responses. Cytokine. 2011;**55**(2):174-180

[31] Liu B, Zhang H, Zhou C, Li G, Fennell A, Wang G, Kang Y, Liu Q, Ma Q. An integrative and applicable phylogenetic footprinting framework for cis-regulatory motifs identification in prokaryotic genomes. BMC Genomics. 2016;**17**:578

[32] Love BC, Hirsh DC. *Pasteurella multocida* produces heat shock proteins in turkeys. Infection and Immunity. 1994;**62**:1128-1130

[33] Masny A, Płucienniczak A. Ligation mediated PCR performed at low denaturation temperatures—PCR melting profiles. Nucleic Acids Research. 2003;**31**(18):e114

[34] Meer RR, Songer JG. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. American Journal of Veterinary Research. 1997;**58**(7):702-705

[35] Mellata M. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. Foodborne Pathogens and Disease. 2013; **10**:916-932

[36] Mian MAR, Hopkins AA, Zwonitzer JC. Determination of genetic diversity in tall fescue with AFLP markers. Crop Science. 2000;**42**:944-950

[37] Mueller PR, Would B. *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. Science. 1989;**10, 246**(4931):780-786

[38] Mueller PR, Wold B, Garrity PA. Ligation-mediated PCR for genomic sequencing and footprinting. Current Protocols in Molecular Biology. Chapter 15:Unit 15.3; 2001

[39] O'Shea BS, Khare K, Bliss P, Klein TA, Ficht LG, Adams, Rice-Ficht AC. Amplified fragment length polymorphism reveals genomic variability among *Mycobacterium avium* subsp. paratuberculosis isolates. Journal of Clinical Microbiology. 2004:3600-3606

[40] Paju S, Goulhen F, Asikainen S, Grenier D, Mayrand D, Uitto V. Localization of heat shock proteins in clinical Actinobacillus actinomycetemcomitans strains and their effects on epithelial cell proliferation. FEMS Microbiology Letters. 2000;**182**:231-235

[41] Prod'hom A, Guilhot C, Gutierrez MC, Varnerot A, Gicquel B, Vincent V. Rapid discrimination of Mycobacterium tuberculosis complex strains by ligation-mediated PCR fingerprint analysis. Journal of Clinical Microbiology. 1997;**35**:3331-3334

[42] Raue R, Schmidt V, Freick M, Reinhardt B, Johne R, Kamphausen L, Kaleta EF, Muller H, Krautwald-Junghanns ME. A disease complex associated with pigeon circovirus infection, young pigeon disease syndrome. Avian Pathology. 2005;**34**:418-425

[43] Sabat E, Rose P, Hickey WJ, Harkin JM. Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Applied and Environmental Microbiology. 2000:844-849

[44] Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: CSH Laboratory Press; 1989

[45] Singh I et al. Molecular mimicry between HSP 65 of *Mycobacterium leprae* and cytokeratin 10 of the host keratin; role in pathogenesis of leprosy. Cellular Immunology. 2012;**278**(1-2):63-75

[46] Singh B, Gupta RS. Conserved inserts in the Hsp60 (GroEL) and Hsp70 (DnaK) proteins are essential for cellular growth. Molecular Genetics and Genomics. 2009;**281**:361-373

[47] Sinha K, Bhatnagar R. Gro EL provides protection against *Bacillus anthracis* infection in BALB/c mice. Molecular Immunology. 2010 Nov-Dec;**48**(1-3):264-271

[48] Skar CK, Kruger PG, Bakken V. Characterisation and subcellular localisation of the GroEL-like and DnaK-like proteins isolated from *Fusobacterium nucleatum* ATCC 10953. Anaerobe. 2003;**9**:305-312

[49] Stathopoulos C, Provence DL, Curtiss R. Characterization of the avian pathogenic *Escherichia coli* hemagglutinin Tsh, a member of the immunoglobulin A protease-type family of autotransporters. Infection and Immunity. 1999;**67**:772-781

[50] Studier FW. Protein production by auto-induction in high-density shaking cultures. Protein Expression and Purification. 2005:207-234

[51] Sunnucks P, Wilson AC, Beheregaray LB, Zenger K, French J, Taylor AC. SSCO is no so difficult: The application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Molecular Ecology. 2000;**9**(11):1699-1710

[52] Tobian AA, Canaday DH, Harding CV. Bacterial heat shock proteins enhance class II MHC antigen processing and presentation of chaperoned peptides to CD4+ T cells. Journal of Immunology. 2004;**173**:5130-5137

[53] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America. 1979;**76**:4350-4354

[54] Uma B, al e. Antibiotic sensitivity and plasmid profiles of *Escherichia coli* isolated from pediatric diarrhea. Journal of Global Infectious Diseases. 2009;**1**(2):107-110

[55] Urban-Chmiel R. Wpływ podwyższonej temperatury na ekspresje˛ białek szoku termicznego (Hsp70) u terenowych szczepów *M. haemolytica* serotyp 1. Medycyna Weterynaryjna. 2006;**62**:801-803

[56] Walter S, Buchner J. Molecular chaperones—Cellular machines for protein folding. Angewandte Chemie (International Ed. in English). 2002;**41**(7):1098-1113

[57] Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, et al. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research. 1995;**23**(21):4407-4414

[58] Yamaguchi H et al. Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*. Journal of Medical Microbiology. 1996;**45**(4):270-277

[59] Yin X, Stam P, Dourleijn CJ, Kropff MJ. AFLP mapping of quantitative trait loci for yield-determining physiological characters in spring barley. Theoretical and Applied Genetics. 1999;**99**:244-253

[60] Young JC, Moarefi I, Hartl FU. HSP90: A specialized but essential protein-folding tool. The Journal of Cell Biology. 2001;**154**(2):267-273

[61] Żaczek A, Brzostek A, Wojtasik A, Sajduda A, Dziadek J. Comparison of ligation-mediated PCR methods in differentiation of *Mycobacterium tuberculosis* strains. Vol. 2014. Hindawi Publishing Corporation, BioMed Research International; 2014. Article ID 782071, 4 pp. http://dx.doi.org/10.1155. DOI: 10.1155

[62] Hugh-Jones ME, de Vos V. Anthrax and wildlife. Revue Scientifique et Technique. 2002;**21**:359-383. DOI: 10.20506/rst.21.2.1336

[63] Spahr C, Knauf-Witzens T, Vahlenkamp T, Ulrich RG, Johne R. Hepatitis E virus and related viruses in wild, domestic and zoo animals: A review. Zoonoses and Public Health. 2018;**65**:11-29. DOI: 10.1111/zph.12405