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EMIL HOLODA, LEON SABA, HANNA BIS-WENCEL,
IVAN MIKULA, BOŻENA NOWAKOWICZ-DĘBEK

Usability of PCR Technique for Salmonella enteritidis

Przydatność techniki PCR w identyfikacji *Salmonella enteritidis*

Detection of the presence of salmonella in food has been one of the top priority and topical tasks of veterinary medicine. The costs of processing the contaminated food are still high. The most frequently diagnosed species in salmonellosis in Slovakia are *Salmonella typhimurium* and *Salmonella enteritidis* (M i č k o v, 4; M i k u l a et al., 5; O n d r a š o v i č et al., 6; O n d r a š o v i č o v a et al., 7; O w o i g b e et al., 8).

Bacterial diagnostics uses a number of methods from conventional bacterial cultivation examinations through radioimmunological analyses (G o n d o l et al., 3) up to DNA methods. One of the DNA methods which has been used increasingly is the PCR method. The advantages of this method are the simplicity of detection, decreased time needed for diagnostics and high specifity. The present study focused on standardization of individual steps in diagnostics of bacterial species *Salmonella enteritidis*.

MATERIAL AND METHODS

BACTERIAL STRAINS

The following bacterial strains were used in our study: *Salmonella enteritidis* 225 (gift from Dr. N a g y, Hungary), field isolant *Salmonella typhimurium* 4/5, plasmid – free apathogenic strain *E. coli* HB 101 and *Proteus vulgaris*. The strains were cultivated in BHI medium overnight at 37°C at shaking.

ISOLATION OF DNA

The bacterial cells obtained from the overnight culture (1.5 ml) by centrifugation (14000 g, 2 min) were rinsed with STE buffer and resuspended in the solution of 0.5% SDS, Proteinase K, to obtain final concentration of 100 $g.ml^{-1}$. After 1 hour incubation at 37°C, 100 l of 5 M NaCl and 80 l of CTAB/NaCl were added and the mixture was incubated at 65°C for 10 min. The samples were first extracted with the mixture chloroform/ isoamyl alcohol and then with phenol/ chloroform/ isoamyl alcohol, precipitated with two aliquot volumes of ethanol and rinsed with 70% ethanol. The sediment obtained by centrifugation was resuspended in TE buffer, treated with RN-ase, rinsed with ethanol and repeatedly resuspended in TE buffer.

REACTION CONDITIONS

The PCR reaction was carried out using AmpliTaqDNA polymerase (Perkin Elmer) in the amount of 0.5 U, 2 mM dNTPs, PCR buffer (Perkin Elmer) with $MgCl_2$ in the total volume of the reaction mixture equal to 100l. To prevent the evaporation, the reaction mixture was overlayed with paraffin oil. The PCR conditions were tested in selected temperature range in relation to time as follows: 1.The preliminary denaturation step, 94-95°C for 1-4 min. 2.The denaturation step, 92-95°C. 3.The anneaing step 52-60°C. 4.The extension step at 70-73°C in the range 0,3-0,6 min while the steps 2-4 were repeated 25,30 or 35 times. 5.The last extension step at 72°C for 2.5 min.

The concentrations of $MgCl_2$ in the reaction buffer was tested for values 1.0; 1.5; 2.0 and 2.5 mM.

The detection of *Salmonella enteritidis* was carried out using species – specific primers: 405 5-AAA GTG ATG CCT TCT GCA TC – 3 and a reverse primer: 407 5 – GCC GTA CAC GAG CTT ATA GA – 3. The primers were sythesized on ID – DLO (Lelystad) according to Wood et al. (9).

THE PREPARATION OF SAMPLES FOR PCR DETERMINATION

To achieve lysis of bacterial cells and the release of DNA, 0,5 ml volume of the sample was placed for 5 min to a boiling water bath and then centrifuged at 14 000 g at 4°C for 5 min. The PCR was carried out using 1 l or 5 l of the supernatant obtained.

AGAROSE GEL ELECTROPHORESIS

The PCR products were identified by means of agarose gel electrophoresis using 2% agarose gel in electrophoretic TAE buffer. DNA was visualised by staining with ethidium bromide (concentration 0.5 $g.ml^{-1}$) under UV light. λ DNA cleaved by restriction endonucleases HindIII and EcoRI (A u s u b e l et al., 1) was used as molecular weight standards.

RESULTS AND DISCUSSION

The 66PCR conditions are affected mostly by primers, the type of polymerase and thermocycler. According to papers published by different authors, the condi-

Table 1. Temperature and applied time in researches

Temperature (°C)	Time (min)	N° of cycles
95	2	1
95	0,3	
52	0,3	25
72	0,3	
72	2,5	1

tions used by them to reach the same final product differed considerably (1, 2, 3). Our conditions were verified using reference laboratory strains *Salmonella enteritidis* 225, *Salmonella typhimurium* 4/5 and *E. coli* HB 101.

When using the species – specific primers 405 5 – AAA GTG ATG CCT TCT GCA TC – 3 and 407 5 – GCC GTA CAC GAG CTT ATA GA – 3, fragment of molecular weight 602 kb (W o o d, 9) was obtained in the positive case. We tested different time ranges, from 0.1 to 0.6 min, for individual steps and searched for optimum concentration of Mg^{+2} ions in parallel testing 1mM; 1.5 mM; 2.0 mM and 2.5 mM concentrations. The quality of the final product was tested by agarose gel electrophoresis evaluating only one band in the assumed zone of 620 kb. The times specified in the Table for individual steps were sufficient at the concentrations

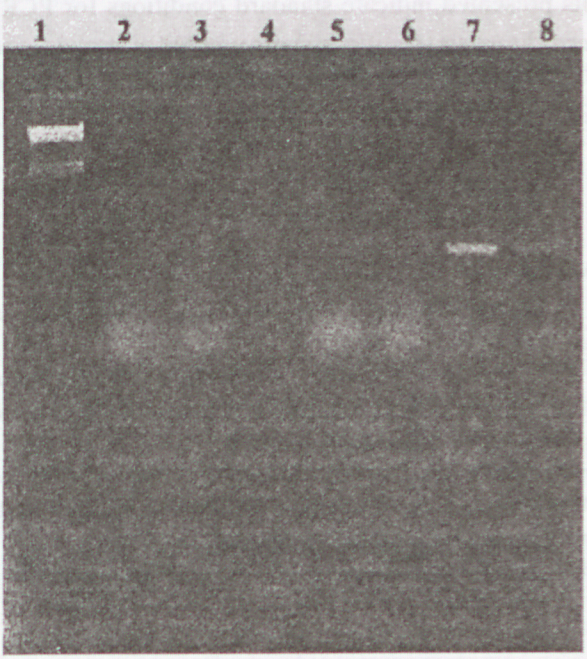

Fig. 1. Agarose gel electrophoresis, from left to right: Line No. 1: molecular weight standard; 2: *E.coli;* 3: *Salmonella typhimurium*; 4: negative control; 5: *Proteus vulgaris;* 6: mixed culture free of *Salmonella enteritidis*; 7: *Salmonella enteritidis*; 8: mixed culture with *Salmonella enteritidis*

1.5 mM or 2.0 mM $MgCl_2$ in the reaction buffer. The positive result was obtained only for DNA isolated from the strain *S. enteritidis*. In the case of DNA isolated from the strains *S. typhimurium* and *E. coli* the results obtained were negative.

The specifity was verified by means of a mixed culture of bacterial strains investigated consisting of *Salmonella typhimurium, Samnonella enteritidid, E. coli* and *Proteus vulgaris*. One ml of mixed culture of final concentration 10^5CFU was added to 9 ml of saline physiological solution. The examination was carried out immediately after mixing and after 24 hours of storage at 4°C at room temperature. Before the CPR, the samples were exposed to 100°C for 15 min and centrifuged at 4°C. One µl of so prepared supernatant was used for analysis. Negative results were obtained for samples in which no *Salmonellae* were present and for the control sample (Figure). All samples in which *Salmonella enteritidis* were present with primers 405 and 407 provided positive results in PCR (Figure, lines 7 and 8). Similar results were obtained for samples which were processed immediately and those processed after 24 hours of storage at 4°C and room temperature.

CONCLUSIONS

1. The results presented indicate standard conditions for PCR diagnostics of *Salmonella enteritidis* using specific primers without nonspecific reactions.

2. It was confirmed that PCR method is supremely sensitive and specific for RNA *S. enteritidris* detection.

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STRESZCZENIE

Praca przedstawia wyniki uzyskane zarówno z wyizolowanych szczepów *Salmonella enteritidis* metodą PCR przy izolacji DNA, jak i mieszaniny kultur bakteryjnych *S. typhimurium, Escheria coli, Proteus vulgaris*. Działania badawcze skupiły się na maksymalnym skróceniu czasu potrzebnego do identyfikacji spodziewanego gatunku bakterii.