Polymerase chain reaction detection of caecal bacteria in case of preventive application of *Enterococcus faecium* EK13 against *Salmonella enterica* subsp. *Enteritidis* in chickens

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ABSTRACT

The polymerase chain reaction (PCR) was used in combination with microbiology methods for identifying faecal bacteria. The changes were monitor in the bacterial flora of chicken's caecum after preventive peroral application of *Enterococcus faecium* EK13 and consecutive infection with *Salmonella enterica* subsp. *Enteritidis.* The preventive treatment with *Enteroccocus faecium* EK13, reduced the numbers of *S. enterica* subsp. *Enteritidis* in chicken excreta after 5 days post infection (pi) and in the caecum after 7 days pi. The PCR products 243 bp for *Sallmonella* spp. and 550 bp for *Ent. faecium* were amplified. The detection of *Salmonella* spp. required only simple boiling of the samples. On the contrary, for reliable detection of *Ent. faecium,* DNA isolation with commercial avalaible kit was required. The high sensitivity of PCR was confirmed and the exptected specific DNA fragments could be amplified from dilutions containing ten to one CFU (Colony Forming Units).

KEY WORDS: enterococci, *Salmonella,* PCR, chicken

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INTRODUCTION

Microbial probiotics have been reported to have many beneficial effects when they are used in animal feeds. One of these effects includes competitive exclusion of pathogens (Morishita et al., 1997; Haschke et al., 1998). Most probiotic bacteria are of intestinal origin and belong to genera *Bifidobacterium* and *Lactobacillus*. Strains from other genera that have been used as probiotics include also *Enterococcus faecium* (Holzapfel et al., 1998). *Enterococci* strains, including *Enterococcus faecium* and *Enterococcus faecalis* are known to produce membrane-active peptides with antimicrobial activity - enterocins (Aymerich et al., 1996; Casaus et al., 1997). The enterocins are generally active against other *Enterococci*, *Listeria monocytogenes,* other lactic acid bacteria as well as *Clostridium* spp. (Giraffa, 1995; Franz et al., 1996). The strain of *Enterococcus faecium* EK13 used in the study was isolated from cattle dung water and it produces the same bacteriocins to those produced by *Enterococci* isolated from food (Marekova et al., 2003).

Genus *Salmonella* represents Gram-negative, facultative, intracellular parasites that invade the mucous membrane and are spread primarily by faecal oral transmission. After *per oral* infection bacteria attach to and enter the epithelial cells of the intestinal *villi*, later on lyse them and they are found in the *lamina propria* or inside the macrophage-like cells (Desmidt et al., 1998). There are more than 2200 different *Salmonella* serotypes (Peltzer, 1989). In commercial chicken flocks, the infection with *Salmonella enteritidis* phage type 4 could be a potential source of human infection (Chart et al., 1990).

The traditional methods for identifying faecal bacteria include various culture techniques, bacteriological isolations, biochemical tests, morphological examination, and analysis of volatile and non-volatile fatty acid production. These methods are time consumming and sometimes cannot distinguish the bacteria on the species level (Bej et al., 1990: Kreader, 1995). Molecular methods, such a PCR have been used to detect probiotic strains (Lucchini et al., 1998). PCR allows the rapid and specific detection of a wide range of bacterial species.

The aim of the study was to evaluate the efficacy and sensitivity of the PCR detection in combination with microbiology techniques.

MATERIAL AND METHODS

Animals

A total of 75 three-day-old chickens strain Isa Brown were included in the experiment. The chickens were kept in standard breeding conditions.The pen was

lit continuously and the optimal temperature was maintained for the age of birds (from 32°C to 26°C at the end of experiment). Water and feed, feed mixture for chickens HYD-04 (Tajba, Slovakia), were available *ad libitum*. Feed mixture composition was, %: crude protein 18, ash 8, tassel 5, lysine 0.9, methionine and cysteine 0.7, plus minerals and vitamins. The animals were divided into 3 groups: E - *Ent. faecium* EK13, ES - *Ent. faecium* EK13 and *Salmonella*, S - *Salmonella*. Each group included 25 chickens (20 for sampling + 5 as back-up). Sampling was done on days 2, 5, 7 and 16 after *Salmonella* infection. Five at random choosen chickens from each group were stunned, bled and picked. Excreta and caecum content from each bird were aseptically removed and used for PCR detection and for microbiological examination. The results were statistically analysed by the Student's t-test.

Bacterial strains, culture medium and growth conditions

The cultivation of *Ent. faecium* EK13 in MRS (de Man-Rogosa-Sharpe broth, Becton Dickinson, Cockeysville, Maryland, USA) at 37°C overnight was followed by dilution to 10^8 cfu/ml in doses. The chickens in groups E and ES were inoculated *per os* for 7 consecutive days with 1 ml of culture. The samples were analysed according to standard microbiological dilution method (ISO 15214). After dilution in saline buffer (pH 7.0), the volume 100 µl of the appropriate dilution was spread onto selective M-*Enterococcus* plates as well as nutrient agar (Becton Dickinson, Cockeysville, Maryland, USA) enriched with rifampicin to detect EK13 strain (rifampicin resistant mutant). Plates were incubated at 37°C for 48 h and checked for the growth of the colonies. Samples were spread onto agar plates in duplicates.

Salmonella enterica subsp. *Enteritidis* SL 2/2 isolate, plasmid profile 55 Kb, phage type 4 (kindly provided from Dr. Šišák-Institute of Veterinary Medicine, Brno, Czech Republic) was used for experimental infection of the birds. *Salmonella* strain was cultivated in Brain Heart Infusion Broth (Oxoid, UK) at 37°C for 20 h. Then it was diluted in sterile PBS to have 10^8 cfu/ml in doses. The chickens were infected *per os* in age 10 days with 1 ml of the culture. *Salmonella* spp. was tested using pre-cultivation in Rappaport-Vassiliadis broth (BioMeriux, Mercy 1'Etoile, France) at 37°C for 24 h followed by spreading of appropriate dilutions (100 µl) onto brilliant green agar plates (Becton Dickinson, Cockeysville, Maryland, USA). Plates were incubated at 37°C for 48 h and checked for the growth of the colonies. Samples were spread onto agar plates in duplicates.

The counts are the average of the counts of 5 samples in each group from excreta as well as from caecum.

PCR primers

The sequences of the primer pairs prepared according to Woodford et al. (1997) and used for DNA amplification of *ddl* gene of region of *Ent. faecium* were as follows: 5'-GCAAGGCTTCTTAGAGA-3' and 5'-CATCGTGTAAGCTAACTTC-3' (Invitrogen, USA). The sequences of the primer pairs used for DNA amplification of *invA* gene region of *Salmonella* spp. were as follows: 5'-ACAGTGCTCGTTTACGACCTGAAT-3' and 5'AGACGA CTGGTACTGATCGATAAT-3' (Invitrogen, USA) prepared according to Chiu and Ou (1996). The Gen Bank program BLAST was used to ensure that the proposed primers were complementary with the target species.

Sample preparation and PCR amplification procedure

For *Salmonella* detection, the fresh content of the caecum was collected and pre-cultivated at 37°C for 8 h in 1.5 ml of LB medium (Oxoid, UK). The samples were then centrifuged at 13 000 r.p.m. for 4 min. The cells in the pellets were washed three times with water and finally resuspended in 100 μl of H_2O. The samples were then boiled for 10 min at 100°C and immediately cooled on ice. After 5 min the suspension was centrifuged at 13 000 r.p.m. for 2 min and the supernatant with eluted DNA was collected to the fresh Ependorf tubes. The 5 μl of the supernatant was directly used to the PCR reaction.

Ent. faecium EK13 was detected from selective M-*Enterococcus* plate enriched with rifampicin. Contemporary the detection of the probiotic strain was made in samples obtained from chicken's caecum enriched in MRS (Oxoid, UK) for overnight. The genomic DNA was extracted from 1.5 ml overnight culture using Wizard Genomic DNA Purification System (Promega, USA) according to manufacturer's instructions. The 10 μl of purified genomic DNA was added to the subsequent PCR reaction.

As a negative control template DNA was used *Escherichia coli* DNA isolated by the same way. The DNAs isolated from pure culture of used *S. enterica* subsp. *Enteritidis* and *Ent. faecium* EK13 strains served as positive controls.

PCR mixtum contained 0.5 μM each primer, 0.2 mM each deoxynucleoside (dATP, dTTP, dCTP, dGTP) (Promega, USA), 2.5 mM $MgCl_2$ (Promega, USA), 1 × PCR buffer (Promega, USA). 1.25 U Taq polymerase (Promega, USA) and H_2O to the total volume of 50 μl. The amplification conditions for *Salmonella* spp. detection were as follows: initial denaturation at 94°C for l min. 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, final elongation at 72° for 10 min. For *Ent. faecium* detection, the conditions were as follows: initial denaturation at 95°C for 2 min. 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, final elongation at 72°C for 10 min. For all samples we used a Techne PTC termocykler (Techne, UK). The PCR products (10 μl of each) were

separated by electrophoresis in 1% agarose gels buffered with 1X TAE (Merck, Germany) containing 0.5 µg/ml ethidium bromide (Promega, USA). The gels were photographed and analysed with Kodak EDAS 120 system (Kodak, USA). The molecular mass standard (Promega, USA) was used according to the manufacturer's instructions and showed one additional band at 1500 bp and 10 bands at 1000 to 100 bp.

RESULTS

Following the microbiological examination, the differences were recorded between groups with applied probiotic strain and pathogen alone. The numbers of *S. enterica* subsp. *Enteritidis* decreased in the excreta and in the caeca content of treated chickens. After 16 days pi, the total counts *of Salmonella* in the excreta, were significantly reduced (P<0.05) in *Enterococci* treated group (ES). In the caecum content, *Salmonella* counts were reduced on 61% of untreated group (S) (Table 1). The total count of applied *Enterococci* slightly decreased. They were stabilized on 4.5 1og10 cfu/g one week after infection with pathogen. The similar counts were recorded also 16 days pi (Table 1).

The PCR amplified products of both used strains were present in the samples from the caeca during the entirely experiment. According to PCR diagnosis, the number of chickens with detected pathogen in the caecum was decreased from 100% 2 days pi to 60% at the end of the experiment in the non-treated group (S). In the ES group the recorded decrease of *Salmonella* excretors was similar. The PCR sensitivity was one CFU in pure *Salmonella* culture (Figure 1).

TABLE 1

The count of *Salmonella enterica* subsp. *Enteritidis* in chicken's excreta and caecum in 1og10 cfu/g and the counts of *Ent. faecium* EK 13 in excreta and caecum of chickens infected with *Salmonella* (ES - preventive *Ent. faecium* EK13 treatment before *Salmonella* infection, S - *Salmonella* infection alone, the same lower case characters showing the significance on the level P<0.05)

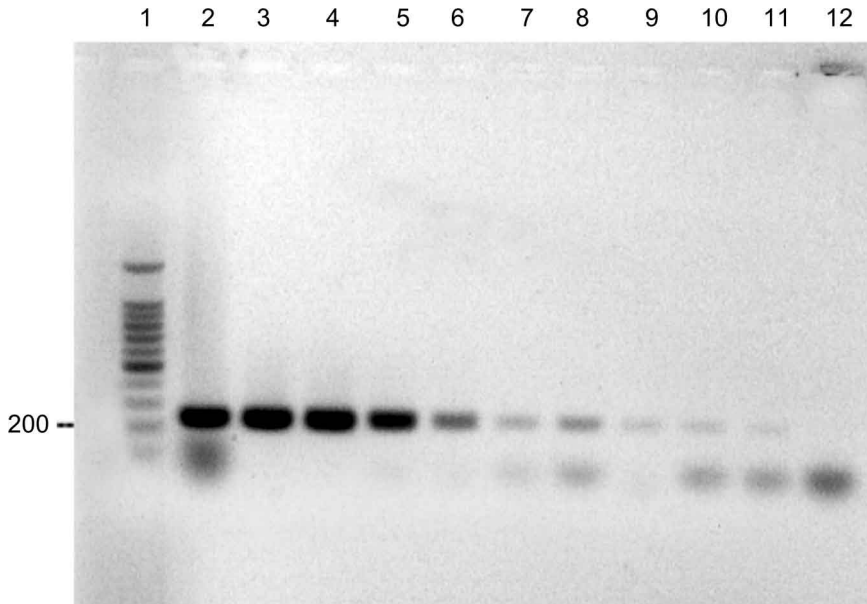| Group/ Bacteria	Excreta, log10 cfu/g				Caecum, log10 cfu/g			
	2d pi	5d pi	7d pi	16d pi	2d pi	5d pi	7d pi	16d pi
ES/ *Salmonella*	5.3 ± 1.1^a	4.7 ± 1.3	4.6 ± 1.7	3.4 ± 1.2^b	5.6 ± 1.3	5.9 ± 1.4	5.4 ± 1.2	4.0 ± 1.3
S/ *Salmonella*	3.2 ± 0.9^a	5.6 ± 1.7	$5.0 + 0.0$	6.3 ± 1.2^b	4.5 ± 1.3	4.3 ± 1.2	5.6 ± 1.6	6.5 ± 2.1
ES/ *Ent.faecium*	6.9 ± 1.4	5.9 ± 1.2	4.4 ± 1.2	4.5 ± 0.3	6.3 ± 2.5	5.5 ± 1.8	4.4 ± 2.5	4.7 ± 2.5

Figure 1. PCR sensitivity test for pure culture of *S. enterica* subsp. *Enteritidis. PCR* products (243 bp) were separated in a 1% agarose gel containing ethidium bromide. Lane 1 contains 100bp DNA ladder. Lanes 2-11 contain the DNA samples extracted from *S. enterica* subsp. *Enteritidis* cultures with 10-fold decreasing cell concentrations ranging from 10^9 to one cell in ml. Lane 12 represents the negative control
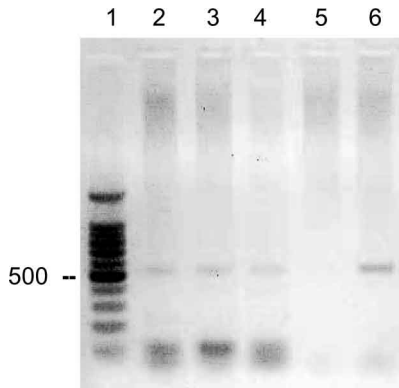

Figure 2. The comparison of isolated DNA of *Ent. faecium* EK 13 from different sources. Lane 1 contains 100 bp DNA ladder. Lane 2 - culture from caecum (E group) after MRS enrichment, lane 3 - culture from caecum (ES group) after MRS enrichment, lane 4 - culture from rifampicin plate after enrichment, lane 5 - negative control, lane 6 - pure culture of EK 13 from glycerin stock after MRS enrichment (positive control)

The *Enterococci* specific amplified products from all sources were compared. They showed the same specific size of PCR fragment - 550 bp in 1% agarose gel analysed by Kodak EDAS 120 system (Kodak - USA) (Figure 2).

DISCUSSION

To be of benefit to an animal, a probiotic organism must have an impact on the microbiology of the gut. The EK13 strain of *Ent. faecium* was primarily isolated from cattle dung water (Lauková et al., 1998) and has been shown to produce *entA* (Mareková and Lauková, 2001). The inhibitory effect of the *entA*-producing EK13 strain of *Ent. faecium* against *S. düsseldorf* SA13 strain, was clearly shown in the model of gnotobiotic Japanese quails (Lauková et al., 2003).

The principal site of *Salmonella* multiplication is the digestive tract, particularly caecum, which may result in widespread contamination of the environment (Popiel and Turnbull, 1995). The frequency of caecal and organ colonization by *S. enteritidis* challenge observed by Guillot et al. (1995) and Duchet-Suchaux et al. (1997) differ between chicken lines. The preventive application of *Ent. faecium* EK13 decreased the numbers of *Salmonella* spp. in the caeca and excreta. Especially, the counts of pathogen were reduced after 16 days pi when compared to group with *Salmonella* infection alone. This resulted in more rapid decrease of *Salmonella* spreading and its presence in the flocks.

The used high dose of *Salmonella* was choosen to indicate the possible inhibitory effect of *Ent. faecium* EK13. In farmer's conditions, lower infection dose and the stable numbers of *Enterococci* recorded in the caecum could avoid the secondary infection or the infection at all. To complete comprehensive studies on the effect of feed additives and applications of probiotic bacterial strains on the composition of intestinal microflora, rapid, sensitive, and specific methods for the detection of intestinal microbes are needed.

Crucial for DNA-techniques is the extraction of DNA from the cells. Gram-negative bacteria are easily disrupted by physical influence (e.g., boiling) and sufficient amounts of DNA are extracted. According to Molander et al. (2002), traditional techniques such as boiling or enzymatic cell lysis followed by proteinase K gave detection levels in the range of hundreds to thousand of *Ent. faecalis* per sample. The simple boiling of *Ent. faecium* EK13 cultures did not result in reliable level of diagnosis. The DNA isolation protocol has to be used in this case.

Using conventional culturing identification methodology on plaque samples, Loesche et al. (1992) reported a detection level of $\geq 2 \times 10^3$ cells. Zambon and Haraszthy (1995) detected 10^4-10^5 cells using non-selective media and 10^3 cells

when selective media were used. Although *Enterococci* easily grow on selective media, PCR might be the slightly superior technology regarding the detection level. The reached level of sensitivity in our experiment was between ten to one CFU.

On the contrary, PCR does not differentiate dead from viable cells. In some cases, especially when growth and biological activity of the artificially applied microbes is observed, it could be a disadvantage. The PCR sensitivity, when made directly from intestinal content, can be decreased because of presence many compounds in intestinal fluids, which can inhibit the PCR.

The combination of common microbiological isolation techniques with PCR "on plate" and after enrichment could be a solution. This was the reason to use such method in our experiment for the detection of used bacterial strains. We did not use the specific set of primers for the detection of *Ent. faecium* EK13. The specificity of the primers was limited for the *Ent. faecium* species. The combination of PCR and microbiological methods using selective media with antibiotics allowed us to improve the specificity of detection. It is useful especially in cases when the correct and specific sequences needed for primer design are unknown, or the desired PCR fragments have similar or same size and the sequencing of the fragments is not possible.

The experiment showed inhibition effect of *Ent. faecium* EK13 against *S. enterica* subsp. *Enteritidis* in chickens. The high sensitivity PCR detection was successfully combined with microbiological methods. The possible disadvantages of both methods were minimized with their effective combination.

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STRESZCZENIE

Zastosowanie łańcuchowej reakcji polimerazy w detekcji bakterii jelita ślepego po zapobiegawczym traktowaniu *Enterococcus faecium* EK13 przeciwko *Salmonella. enterica* subsp. *Enteriditis* u kurcząt

Identyfikację bakterii jelita ślepego u kurcząt przeprowadzono metodą PCR oraz technikami mikrobiologicznymi. Zmiany we florze bakteryjnej jelita ślepego monitorowano po doustnym ochronnym podaniu *Enterococcus faecium* EK13 i jednoczesnej infekcji ptaków bakterią *Salmonella enterica* subsp *Enteriditis*. Obniżenie poziomu *S. enterica* subsp. *Enteriditis* w odchodach stwierdzono w 5 dniu po prewencyjnej infekcji *Ent. faecium* EK13, podczas gdy w jelicie ślepym efekt ten wystąpił po 7 dniach od jej podania. Z DNA bakterii *Salmonella* spp.. zamplifikowano produkt o długości 243 pz, podczas gdy z DNA bakterii *Ent. faecium* otrzymano produkt o długości 550 bp. Wykrycie bakterii *Salmonella* spp. wymagało tylko zagotowania prób, podczas gdy precyzyjna detekcja *Ent. faecium* wymagała zastosowania kitów komercyjnych do izolacji DNA. Wysoka czułość reakcji PCR pozwala na amplifikację specyficznych fragmentów DNA z niskich (10 :1) rozcieńczeń CFU.