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Optimalization of *Salmonella* detection in chickens' caecum using PCR method (short communication)

Abstract

PCR analysis is regarded to be a quick and reliable method of *Salmonella* detection in food and animal samples. The mode of sampling and sample preparation influence the sensitivity and accuracy of the analysis. The 10 days old, clinically healthy *Salmonella*-free chickens were experimentally infected with *Salmonella enterica* subsp. *Enteritidis* strain. Five randomly selected birds were euthanized at intervals from 2 days to 16 days post-infection (pi). DNA detection with polymerase chain reaction (PCR) as a mean of identifying *Salmonella* infection in chickens' caecum was used. The caecal sample was diluted into LB medium and incubated to eliminate inhibitory compounds and to allow enrichment of the bacteria. The DNA was extracted from cultures by boiling method. The pair of primers used was those directed at the *invA* gene. As expected a 243 bp fragment DNA was amplified from extracted DNA by PCR. *Salmonella* DNA was detected throughout the entire test period. The number of chickens containing salmonellae in caecum varied during the 16 days post-infection between 100% and 60%. The stated sensitivity of PCR reaction was 1 CFU of used strain in pure culture. The faeces and caecum content were simultaneously examined by conventional culture procedure. The microbiological examinations showed the presence of salmonellae in faeces during the entirely experiment. The results of this study confirm that PCR is a useful tool for the detection of *Salmonella* infection in poultry.

Key Words: *Salmonella*, chicken, PCR

Zusammenfassung

Titel der Arbeit: **Optimierung der Salmonellendetektion im Blinddarm von Hühnern mit der PCR Methode** (Kurzmitteilung)

Die PCR (Polymerasekettenreaktion) Analyse wird als eine schnelle und zuverlässige Methode der Salmonellen-detektion in Nahrungsmitteln und Proben aus Tiermaterial angesehen. Der Modus der Probenentnahme und die Probenvorbereitung beeinflussen die Empfindlichkeit und die Genauigkeit der Analyse. Klinisch gesunde, salmonellen-freie Hühner wurden im Alter von 10 Tagen experimentell mit *Salmonella enterica* subsp. *Enteritidis* infiziert. Ingesamt 5 nach dem Zufallsprinzip ausgewählte Tiere sind im Abstand von 2 bis 16 Tagen nach der Infektion (pi) euthanasiert worden. Der DNA-Nachweis erfolgte mit Hilfe der PCR als Erkennungsgrundlage einer Salmonelleninfektion im Hühnerblinddarm. Die Caecalprobe wurde in LB Medium verdünnt und bebrütet, um Hemmstoffe zu beseitigen und eine Bakterienanreicherung zu ermöglichen. Die Extraktion der DNA aus den Kulturen erfolgte durch Kochen. Ausgewertet wurden die Sequenzen der Primerpaare aus der *invA* Genregion der Salmonellen. Wie zu erwarten, wurde das 243 bp Fragment DNA von extrahierter DNA durch PCR verstärkt. Salmonellen DNA ist während der gesamten Testperiode gefunden worden. Die Zahl der Hühner, bei denen Salmonellen im Blinddarm nachgewiesen wurden, schwankte im Verlauf der 16 Tage nach der Infektion zwischen 100% und 60%. Die festgestellte Empfindlichkeit der PCR Reaktion betrug 1 CFU des verwendeten Strains in der Reinkultur. Die Exkremente und der Blinddarminhalt wurden gleichzeitig durch übliche Kulturverfahren überprüft. Die mikrobiologischen Kontrollen ergaben, dass während des gesamten Versuches Salmonellen in den Exkrementen enthalten waren. Die Ergebnisse der Untersuchungen bestätigen, dass PCR für die Erkennung einer Salmonelleninfektion beim Geflügel von Nutzen ist.

Schlüsselwörter: *Salmonella*, Geflügel, PCR

Introduction

The microflora in the intestinal tract is an extremely complex relatively stable ecological community that is populated e.g. in humans by an excess of 10^{11} bacterial cells per g of content and contains more than 400 bacterial species in the colon (DRASAR and ROBERTS, 1990). Genus *Salmonella* represents Gram-negative, facultative, intracellular parasites that invade the mucous membrane. There are more than 2200 different *Salmonella* serotypes (PELZER, 1989). Bacterial food-borne zoonotic infections are the most common cause of human intestinal disease. *Salmonella* and *Campylobacter* account for over 90% of all reported cases of bacteria-related food poisoning world-wide. Poultry and poultry products have been incriminated in the majority of traceable food-borne illnesses caused by these bacteria, although all domestic livestock are reservoirs of infection (THORNS, 2000).
Salmonella infection in food-producing animals is often clinically not apparent. The principal site of multiplication of these bacteria is the digestive tract, particularly caecum, which may result in widespread contamination of the environment. The pathogenicity of salmonellas depends on the invasive properties and the ability of the bacteria to survive and multiply within cells, particularly macrophages. These properties are the source of vertical transmission which, in the case of survival of the embryo, can result in contamination of a flock or, in the case of embryonic mortality, can result in an explosion of contaminated eggs (HUMBERT and SALVAT, 1997).
The increased frequency with which food-borne bacterial pathogens have been causing recurring outbreaks, sometimes with fatal infections, has led to the development of numerous diagnostic techniques. Conventional isolation methods are, in theory, sensitive enough to detect one cell in a defined food sample. On the contrary, COHEN et al. (1994) observed that microbiological culture was less sensitive than a PCR assay and the results of culture of faeces with less than 10^2 CFU/g were negative.
The conventional methods also require multiple subculturing stages followed by biochemical and serological confirmatory tests which can take up to 7 days. PCR analysis offers several advantages in a single package, including the sensitivity and rapidity. Therefore, the aim of this study was the optimization of the PCR detection of *Salmonella* from the chicken's caecum content.

Material and methods

Animals

A total of 50, one-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. Water and feed – feed mixture for chickens HYD-04 (Tajba, Slovakia), were available *ad libitum*. Sampling was done on days 2, 5, 7 and 16 after *Salmonella* infection. The chicks were stunned, bled and picked. Caecum from each bird was aseptically removed and its content used for PCR detection. Survival of *Salmonella enterica* subsp. *Enteritidis* was controlled by the standard dilution microbiological method.

Bacterial strains, culture medium and growth conditions

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. PT4 strain was cultivated

in Brain (BHI Oxoid, UK) at 37° C for 20 h. Then it was diluted in sterile PBS to have 10^8 CFU/ml in doses. The chicks were infected *per os* in age 10 days. *Salmonella* sp. were tested using pre-cultivation in Rappaport- Vassiliadis broth (BioMeriux, Mercy l'Etoile, France) at 37 °C for 24 h followed by spreading of appropriate dilutions (100 µl) onto Brilliant green agar plates (Becton & Dickinson, Cockeysville, 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.

PCR primers

The sequences of the primer pairs used for DNA amplification of *invA* gene region of *Salmonella* sp. were as follows: 5'-ACAGTGCTCGTTTACGACCTGAAT-3' and 5'-AGACGACTGGTACTGATCGATAAT-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

The fresh content of the caecum was collected and the sample was diluted into 1.5 ml of LB medium and incubated at 37 °C for 8 hours to eliminate inhibitory compounds and also to allow enrichment of the bacteria. Samples were then centrifuged at 16 000 x g for 3 minutes. The cells in the pellets were washed three times with water and finally resuspended in 100 µl of H_2O. Samples were then boiled for 10 minutes at 100°C and immediately cooled on ice. After 5 minutes the suspension was centrifuged at 13 000 r.p.m. for 3 min and the supernatant with eluted DNA was collected to the fresh Ependorf tubes. 5 µl of the supernatant was directly used to the PCR reaction. As a negative control template DNA, *E. coli* DNA isolated by the same way was used. The DNA isolated by boiling method from pure culture used *Salmonella* strain served as positive control. PCR mixture contained 0.5 µM each primer, 0.2 mM each deoxynucleoside (dATP, dTTP, dCTP, dGTP) (Promega, USA), 2.5 mM $MgCl_2$ (Promega, USA), 1x PCR buffer (Promega, USA), 1.25 U Taq polymerase (Promega, USA) and H_2O to the total volume of 50 µl. The amplification conditions were as follows - initial denaturation at 94°C for 1min, 30 cycles of 94°C for 30 s, 56°C for 30s, 72°C for 2 min, final elongation at 72° for 10 min with a Techne PTC termocykler (Techne, UK). The PCR products (10 µl of each) were separated by electrophoresis in 1 % agarose gel buffered with 1X TAE (Merck, Germany) containing 1 µg/ml ethidium bromide (Promega, USA) at 80V for 45 min. 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.

Sensitivity of PCR detection

A pure bacterial culture of *Salmonella enterica* subsp. *Enteritidis* PT4 $1x10^9$ cell/ml was serially diluted 10-fold to one cell/ml. These cells suspensions were then centrifuged (16 000 x g) for 3 min. The pellets of bacteria were washed three times with H_2O and finally resuspended in 100 µl of water and boiled for 10 min. After centrifugation at 16 000 x g for 3 min, five microliters of each dilution were added directly to the PCR mixture.

Results

The PCR amplified product of 243 bp was present in the samples from the caeca during the entirely experiment. According to PCR diagnosis, the number of chicks with *Salmonella enterica* subsp. *Enteritidis* PT4 in the caecum content varied during the experiment from 100% to 60% at the end of experiment.
Upon the microbiological examination, the differences between the presence of pathogen in the caecum and in the faeces were recorded (Tab.).

Table
Total counts of *Samonella enterica* subsp. *Enteritidis* PT4 in the caecum and faeces of infected chickens during experiment (total counts are expressed as colony forming units per ml) (Gesamtzahlen von *Samonella enterica* subsp. *Enteritidis* PT4 in Blinddarm und Exkrementen der infizierten Hühner während des Experimentes (Gesamtzahlen werden als Kolonieeinheiten pro ml ausgedrückt))

	Sampling (days post infection)			
Sample	2 days	5 days	7 days	16 days
Faeces	$1.6 \pm 0.9 \times 10^3$	$4.2 \pm 1.7 \times 10^5$	$1.0 \pm 0.0 \times 10^5$	$2.1 \pm 0.2 \times 10^6$
Caecum	$3.5 \pm 1.5 \times 10^4$	$1.8 \pm 0.2 \times 10^4$	$5.7 \pm 1.6 \times 10^5$	$3.0 \pm 0.5 \times 10^6$

In our experiment, the high sensitivity of PCR was confirmed and the expected 243 bp specific DNA fragment could be amplified from dilutions containing one CFU of used *Salmonella* strain (Fig.). However, this level of sensitivity was reached in pure *Salmonella* culture.

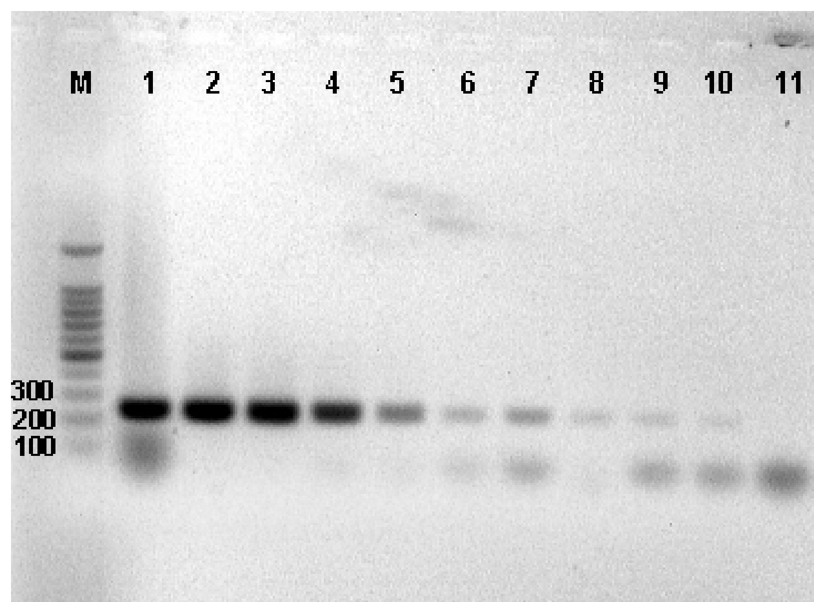

Fig.: PCR sensitivity test for pure culture of *Salmonella enterica* subsp. *Enteritidis. PCR* products (243 bp) were separated in a 1% agarose gel containing ethidium bromide. Lane M contains 100bp DNA ladder (sizes are shown at the left). Numbered lanes contain the DNA samples extracted from *Salmonella enterica* subsp. *Enteritidis* cultures with 10-fold decreasing cell concentrations ranging from 10^9 to one cell ml^{-1} (lanes 1 to 10). Lane 11 represents the negative control (PCR Empfindlichkeitstest für die Reinkultur von *Salmonella enterica* subsp. *Enteritidis*. PCR Produkte (243 bp) wurden in einem 1% Agarosegel getrennt, das Äthidiumbromid enthält. Weg M enthält Strichleiter der DNA 100bp (Größen werden am Link gezeigt). Nummerierte Wege enthalten die DNA-Proben, die von den *Salmonella enterica* subsp. *Enteritidis* Kulturen mit 10-facher abnehmender Zellkonzentration extrahiert werden, die von 10^9 bis eine Zelle ml^{-1} reichen (Wege 1 bis 10). Weg 11 stellt die negative Kontrolle dar)

Discussion

SKOV et al. (2002) observed that the number of faecal excretors declined with time, down to 6% within the first 8 weeks for *S. enteritidis*. The faster decrease of

Salmonella carrier rate recorded in our experiment within first two weeks pi depends presumably on age of the infected chickens. On the contrary to SKOV et al. (2002), in our experiment older chickens were infected to prove the detection ability of the PCR in case of wider bacterial spectrum in the intestine at the age of ten days.
The PCR results are in agreement with the studies using traditional methods for the detection of intestinal microbes in mixed populations. However in studies of WHYTE et al. (2002) the detection of *Salmonella* in raw broiler carcasses using PCR was more sensitive compared to traditional culture methods. SCHRANK et al. (2001) detected only 47 % of all PCR positive samples with the standard microbiological procedure.
The specificity of a PCR based assay for the detection depends on the fidelity of the primers used. The sensitivity of PCR methods can vary. For example, WANG et al. (1996) were able to detect in case of *Bifidobacterium adolescentis* not less than 10000 cells, which makes PCR for quantification of this species not very sensitive.
There is a relentless effort to find new primer sequences which are uniques to the target pathogen. It exists many different primers and approaches for detection of *Salmonella* in food and in faeces from poultry (ABO et al., 1993; FLUIT et al., 1993; COHEN et al., 1994; STONE et al., 1994; JITRAPAKDEE et al., 1995; KWANG et al., 1996).
The crop and the caecum are the major sites of *Salmonella* colonization in the chickens after oral infection (BROWNELL et al., 1970). VAN IMMERSEEL et al. (2002) infected 2-day-old chicks with *Salmonella enterica* subsp. *Enteritidis* and they found bacteria invaded the *lamina propria* of the caecal wall from 12h post-challenge onwards.
These sites are responsible for the spread of *Salmonella* to internal tissue (NAGARAJA et al., 1991). However, differences in the susceptibility of chicken breeds to *Salmonella* spp. infection have been explored (TREBICHAVSKÝ, 1999). POPPE et al. (1993) demonstrated that the course of infection with *S. enteritidis* is dependent on the bacterial strain, the host (age, breed, immune status), the environment (natural infection) and the experimental design (e.g. route of infection, inoculum dose, housing).
In the faeces and intestinal content are presented many inhibition factors as bilirubin, bile salts, enzymes, food residues, products of bacterial metabolism, etc. These compounds can decrease the PCR sensitivity or inhibit the PCR analysis at all (KREADER, 1995; WILDE et al., 1990). Therefore, PCR detection of bacteria in faeces or in intestine content usually requires DNA purification. The DNA isolation and purification procedures are time and finance consuming.
COHEN et al. (1994), who isolated the DNA from faecal samples of hens reached PCR sensitivity level at 1 CFU of *S. enteritidis*/g faeces. PCR sensitivity in our experiment is comparable with these results. Centrifugation and washing steps were sufficient procedures to remove the PCR inhibitors from culture medium. Boiling the bacterial cells and then immediately cooling them on ice was efficient for releasing and denaturing the DNA template. Resulting suspension was directly subjected to the PCR assay without DNA isolation and purification steps.
The advantage of direct PCR from the caecum content is detection both viable and non-viable cells. The disability is the presence of PCR inhibitors as mentioned above. PILLAI et al. (1994) used a rapid sample processing method to detect low numbers of *Salmonella typhimurium*. They were able to detect with *phoP* specific primers fewer

than 100 CFU when pure cultures were employed. When the polymerase chain reaction was run on caecal contents from birds infected with *S. typhimurium* the sensitivity was about 700 CFU detectable in samples.
In this experiment a combined method of *Salmonella* detection was used. Salmonellae are characterised by good multiply abilities in usual media and there are no special needs for cultivation. This fact gave us a possibility to increase the sensitivity of our PCR detection. Under our conditions was able to detect one CFU in pure culture. The method used for sample processing is simple, effective and it provides a sensitive means of detecting salmonellae-specific sequences in mixed caecal microbial population.

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