Application of qPCR Method for Investigation of Plant Colonization by Human Pathogen Bacteria

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Abstract

The consumption of vegetables is very important for prevention cardiovascular diseases and it is recommended by WHO. The fresh vegetables are essential for healthy nutrition and provide minerals and vitamins. The vegetables are mostly consumed raw and it is very important to avoid its microbiological contamination during the production chain. The disease which are caused by human pathogen bacteria are very big problem for public health. These bacteria are able to contaminate fresh vegetables in any part of chain of food production.

The Salmonellosis is the usual foodborne infection which is caused by bacteria *Salmonella spp.* According to U.S. Public Health Service (2009), *Salmonella* is in the second place as causer of foodborne disease in the USA. Approximately, there are 40 000 cases of Salmonellosis in the USA per year (DFBMD 2009).

The most common serovars which are found worldwide are *Salmonella enteritidis* and *Salmonella typhimurium* but others serovars are limited to specific regions in the world (OIE 2005).

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Today, there is lot of methods for detection human pathogen bacteria in food. The conventional methods are generally timeconsuming. The PCR is much faster and with qPCR we can get results only in few hours.The PCR allows increasing speed, sensitivity, specificity of detection of human pathogen bacteria in fresh vegetables.

The aim of this study is application of the qPCR method for detection of *Salmonella enterica* subsp. Welteweden and *Salmonella typhimurium* LT2 in wheat seedlings.

Bacterial strains used in this experiment were *Salmonella enterica* subsp. Welteweden and *Salmonella typhimurium* LT2. The model plant was wheat. The bacterial suspension applied for inoculation seeds was ≈ 10^8 CFU. The inoculated plants left to grow in phitochamber for 3 weeks. The standard PCR was done for *Salmonella* strains. The primers for *Salmonella* were: rfbJ; fliC; fijB; invA, hilA. It was done cloning for getting plasmid with invA gene which was used for preparing standards for qPCR. Isolation *Salmonella* DNA from plants was done using kit. The sequencing of invA isolated from plant samples also was done.

The qPCR was done for DNA samples isolated from wheat root, shoot and substrate liquid inoculated with *Salmonella* strains. The Fluorescence *In Situ* Hybridization was done for inoculated plant samples. It was used specific probes for detection *Salmonella* by CLSM.

The results show that both investigated *Salmonella* strains were able to colonize wheat plants. The number of *Salmonella* DNA copies was 4.01×10^6 per 1 g root (*S. enterica*) and 3.32×10^7 per 1 g root (*S. typhimurium*).

1 Introduction

In recent years, there is an increasing number of outbreaks caused by consumption fresh vegetables which are contaminated with human pathogen bacteria. The application organic fertilizers and contaminated irrigation water are the main reasons of contamination by pathogen bacteria during the food production.

The consumption of vegetables is very important for prevention cardiovascular diseases and it is recommended by World Health Organization. The fresh vegetables are essential for healthy nutrition and provide minerals and vitamins. The vegetables are mostly consumed raw and it is very important to avoid its microbiological contamination during the production chain.

Recently there has been an increasing number of outbreaks caused by contaminated fresh vegetables. According to CDC (Center of Disease Control and Prevention, 2010), the lettuce was the one of the most frequent source of foodborne outbreaks in USA during the 2007.

Today, there are many methods for detecting human pathogen bacteria in food. The conventional methods which are based on cultures are generally timeconsuming and new methods are needed to exceed their performance.

Immunology-based methods for detection human pathogen are very powerful tools and they provide extraction pathogen from bacterial suspension usingantibody coated magnetic beads. The PCR methods give more conclusive results, especially recent advances in PCR technology.Thus, with Real-Time-PCR (RT PCR), we are able to get very precise results only in few hours. Today, the most common methods for human pathogen detection in fresh vegetables are: colony counting technique, PCR and immunology-based methods. The PCR is much faster than other techniques and it takes approximately 6 to 24 hours to get result and this method does not need and include any previous enrichment steps. On the other hand, with RT PCR we can get results faster, only in few hours.

The general (conventional) PCR protocol for detection human pathogen bacteria in samples includes: denaturation of DNA, annealing of sequence specific primers, extension by polymerase 25–40 cycles. The PCR product can be analysed by gel electrophoresis or DNA sequencing. The qPCR is technique for amplification and simultaneously quantification a targeted DNA molecule.The qPCR allows detection and quantification of DNA sequence in real time after amplification cycle. The quantification includes fluorescent dyes which insert with double-stranded DNA during PCR oligonucleotide probes that illuminate after hybridization with complementary DNA and extension. The qPCR is combination of the amplification DNA and quantification of amplified DNA in real time. Also, it is possible to use probes which are labeled with different dyes and they allow quantification and detection of multiple target genes in one PCR reaction.

In comparing with conventional microbiology methods, the PCR technique is much faster and requires less time to achieve precise and valid results. The advantage of PCR is detection of bacteria which are not able to grown in culture. The PCR allows increasing speed, sensitivity, specificity of detection human pathogen bacteria in fresh and ready to eat vegetables.

Today, it is known two qPCR methods, TaqMan and SYBR Green. The TaqMan PCR is based on fluorescent probes which must be selected according to very strict conditions and it cannot be always applied. The SYBR Green qPCR provides fast result compared to others technique and detection is based on binding of SYBR-Green dye into double stranded PCR products. It could be applied without the needing for probes linked to fluorescent molecules.

This study tries to develop protocol for rapid and precise detection of human pathogen bacteria in contaminated plants using the qPCR. The aim of this work is to develop and improve microbiological laboratory analysis of human pathogens using real-time PCR, develop a PCR method and develop a validation of protocol for it. It is very important to establish simple and reliable qPCR method which is using SYBR Green that could be suitable for routine analyses of *Salmonella spp*. in plants and fresh vegetables.

In general, the aim is application of the real time qPCR method for detection of*Salmonella enteric*a subsp. Welteweden and *Salmonella typhimurium* LT2 in wheat seedlings and to get an expertise with pathogen detection methods using real time qPCR techniques.

2 Materials, Methods and Notes

Bacterial strains which are used were *Salmonella enteric*a subsp. Welteweden and *Salmonella typhimurium* LT2. The model plant for inoculation was wheat. The sterile wheat seed was incubated on NB plates at 30° C for 3 days, letting them germinate and plants were grown on quartz sand in sterile conditions. The bacterial suspension which is applied for inoculation seeds was ≈ 10^8 CFU (OD_{600} = 0.7) for both *Salmonella* strains. Before inoculation, the seedlings were washed in sterile H_2O five (5) times and they were kept in bacterial (*Salmonella*) solution 1 hour at 20°C before planting. The inoculated plants left to grow in phytochamber for 3 weeks.

The standard PCR was done for pure culture of *Salmonella enteric*a subsp. Welteweden and *Salmonella typhimurium* LT2. The standard PCR included extraction bacterial (*Salmonella*) DNA using the Genomic DNA From Tissue kit, NucleoSpin Tussue (Machery-Nagel, www.mn-net.com). It was used specific primers for *Salmonella*: rfbJ; fliC; fijB; invA and hilA (for *Salmonella typhimurium* LT2). It was used 16 S PCR Program: Hotstart at 94°C – 5 min.; Denaturating at 94°C – 45 sec.; Annealing at 54°C – 45 sec.; Elongation at 72°C – 45 sec.; Final elongation at 72°C – 5 min.; Store at 4°C – continous (30 cycles). The electrophoresis was done in 1% Agarose in TAE Buffer + 3µl EtBr/100 ml at 120V; 400mA; 100W; 45 minutes.

Also, FISH (Fluorescence *In Situ* Hybridization) was done for pure cultures of *Salmonella enteric*a subsp. Welteweden and *Salmonella typhimurium* LT2. The cultures of *Salmonella* strains incubated overnight in NB Broth at 37°C with shaking. After that, it was done fixation in Paraformaldehyde (PFA) (for G^- bacteria) according to Protocol Fixation Of Bacterial Liquid Cultures. The

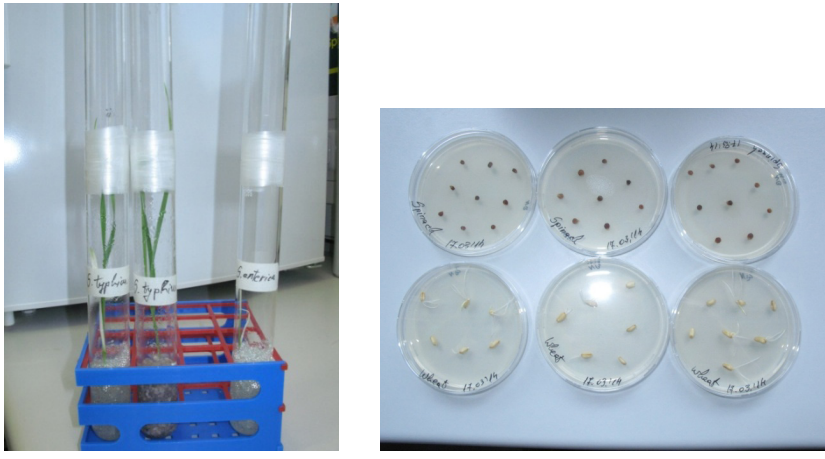

Figure 1: The wheat plant in glass tubes and seed germination in Petri dish.

Primer	Target gene	Primer length (bp)	Sequence	Size of amplified fragment (bp)	*Numbers designate Genbank-EMBL-DDBL ID numbers of the sequences in databases*
RfbJ-s RfbJ-as	*rfbJ*	24 24	5'-CCAGCACCAGTTCCAACTTGATAC-3' 5'-GGCTTCCGGCTTTATTGTTAAGCA-3'	663	AE008792
FliC-s FliC-as	fliC	24 24	5'-ATAGCCATCTTTACCAGTTCCCCC-3' 5'-GCTGCAACTGTTACAGGATATGCC-3'	183	D13689
FljB-s FljB-as	fljB	24 24	5'-ACGAATGGTACGGTCTCTGTAACC-3' 5'-TACCGTCGATAGTAACGACTTCGG-3'	526	AF045151
139-s 141-as	*invA*	26 22	5'-GTGAAATTATCGCCACGTTCGGGCAA-3' 5'-TCATCGCACCGTCAAAGGAACC-3'	284	Malorny at al. (2003)

Table 1: Primers for *Salmonella strains.*

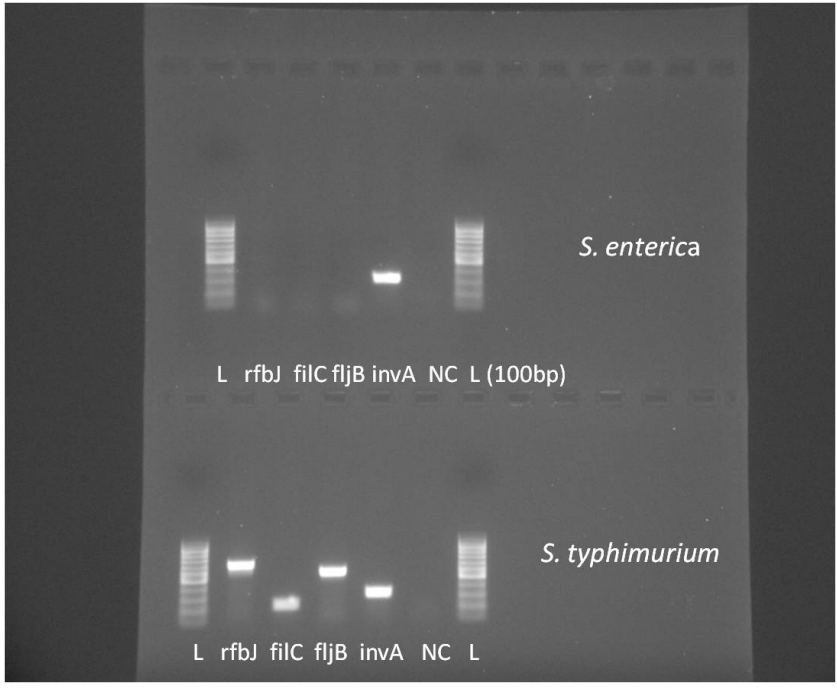

Figure 2: Standard PCR for pure culture of *Salmonella*.

oligonucleotide probes used in this analyze were: Salm-63-Cy3; Gam42a-Fluos; Bet42a-Oligo. The FISH protocol is: add 1–10µl PFA-fixed sample onto glass slide; drying at 46°C; EtOH-dehydration in 50%, 80%, 100% for 3 min. each; air drying; add 8µl hybridization buffer and 1µl probe. The samples were observed by CLSM (confocal laser scanning microscope).

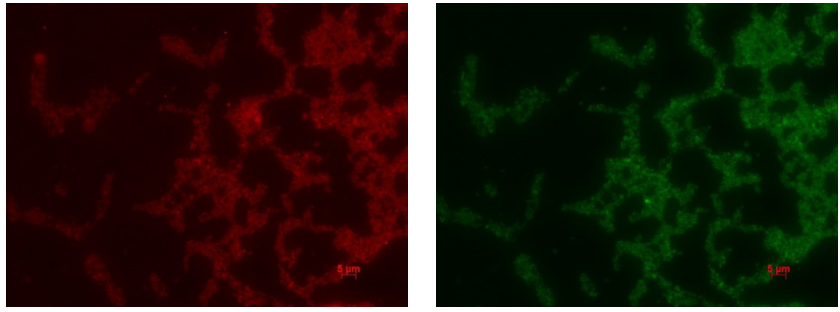

Figure 3: FISH for pure culture of *Salmonella typhimurium* LT2.

qPCR Analyses: In the goal to preparing standards for qPCR, cloning was done to get plasmid of *Salmonella* strains. PCR cloning was done for invA gene at *Salmonella* strains (*Salmonella enterica* subsp. Welteweden and *Salmonella typhimurium* LT2) according to PCR cloning protocol StrataClone PCR Clonong Kit (Stratagene). The protocol includes: Isolation plasmid with invA from *E. coli* competent cells (it is done by kit Plasmid DNA purification according to protocol NucleoSpin Plasmid QuickPure protocol); preparing standards for qPCR (it is calculated a number of moleculs per 1 µl of invA copies). After that, qPCR for DNA samples of *Salmonella* pure cultures was done according to 16 S PCR program and qPCR-samples were run on the gel.

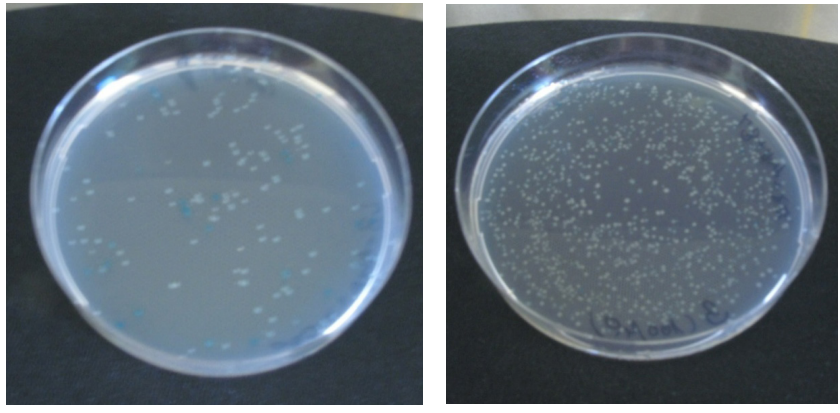

Figure 4: Colonies of transformed competent cells with plasmid of invA.

Isolation of Salmonella DNA from plants: The *Salmonella* DNA was isolated from:wheat root; wheat shoot (stem and leaves) (quartz sand) liquid. The protocol for isolation *Salmonella* DNA from wheat plants and substrate liquid includes: it is taken 0.5 g of plant material and 500 μl of liquid for analyses; the plant root and shoot were crashed with mortar and pestle in liquid nitrogen; after that, DNA from plant material and substrate liquid are further isolated according to FastDNA SPIN Kit for Soil (www.mpbio.com). Standard PCR for DNA isolated from plant and liquid samples was done to check presence of *Salmonella* DNA. The positive control is DNA isolated from *Salmonella* pure culture and negative control is reaction mixture without DNA.

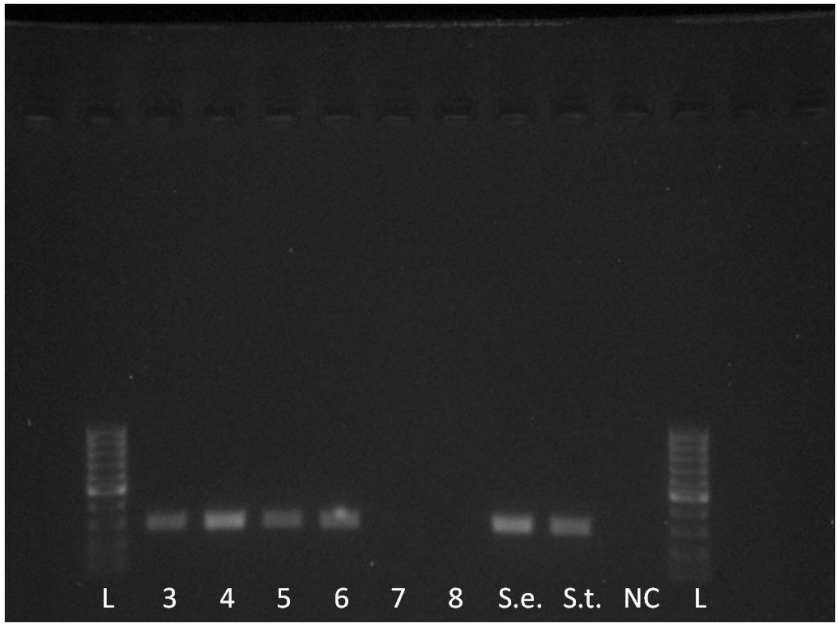

Figure 5: The standard PCR for DNA isolated from plant samples to check presence of *Salmonella* DNA (invA).

Legend: 3–wheat root inoculated by *Salmonella typhimurium* LT2; 4–wheat shoot inoculated by *S. typhimurium* LT2; 5–wheat root inoculated by *Salmonellaenterica* subsp. Welteweden; 6–wheat shoot inoculated by *S.enterica* subsp. Welteweden; 7–substrate liquid inoculated by *S. typhimurium* LT2; 8–substrate liquid inoculated by *S.enterica* subsp. Welteweden; S.e. and S.t.-positive control; NC-negative control.

Also, sequencing of invA isolated from plant samples was done and this analysis consisted of thefollowing: standard PCR; run gel; purification PCR mastermix samples; determination of DNA concentration by NanoDrop; Seq-PCR;

purification Seq-PCR product; putting samples in microtiter plate and doing sequencing.

Finally, the quantitative PCR (qPCR) was done for DNA samples isolated from: wheat root inoculated with *Salmonella typhimurium* LT2; wheat shoot inoculated with *Salmonella typhimurium* LT2; wheat root inoculated with *Salmonella enterica* subsp. Welteweden; wheat shoot inoculated with *Salmonella enterica* subsp. Welteweden; liquid inoculated with *Salmonella typhimurium* LT2 and liquid inoculated with *Salmonella enterica* subsp. Welteweden. The DNA samples were run on the gel.

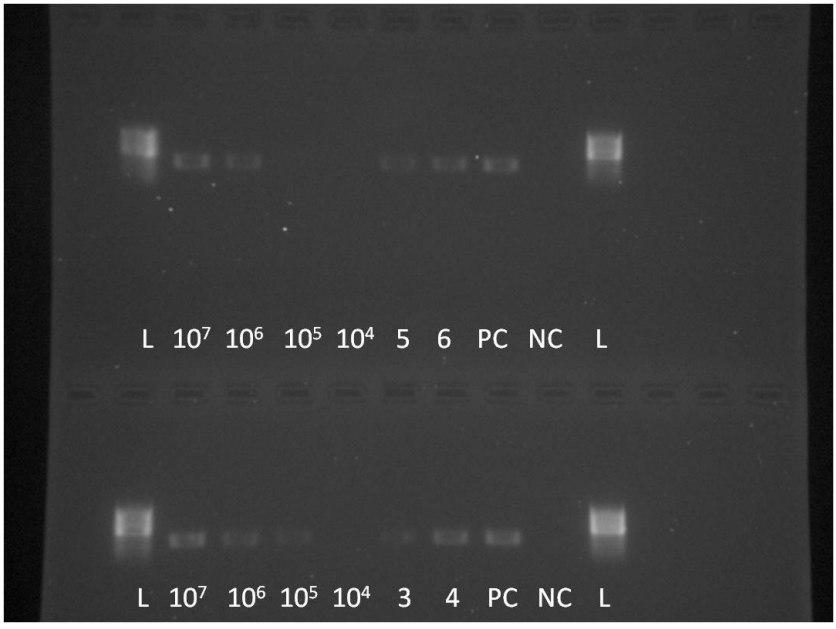

Figure 6: qPCR products of wheat samples inoculated with *Salmonella* strains (3; 4; 5; 6) on the gel.

Legend: 3–wheat root inoculated by *Salmonella typhimurium* LT2; 4–wheat shoot inoculated by *S. typhimurium* LT2; 5–wheat root inoculated by *Salmonellaenterica* subsp. Welteweden; 6–wheat shoot inoculated by *S.enterica* subsp. Welteweden; PC-positive control; NC-negative control.

Also, FISH was done for inoculated plant samples and CLSM analyses and sampleswere: wheat root, stem and leaf. The specific probes which were used for detection *Salmonella* strains by CLSM were: Salm 63 – Cy3 (red); Gam 42 – Fluos (green) and Bet 42 a – Oligo. The FISH analyses were done according to protocol: In Situ Hybridization Protocol for plant material.

The investigated *Salmonella* strains were able to colonize wheat plants. The number of *Salmonella* DNA copies was 4.01×10^6 per 1 g root (*S. enterica*) and 3.32×10^7 per 1 g root (*S. typhimurium*).

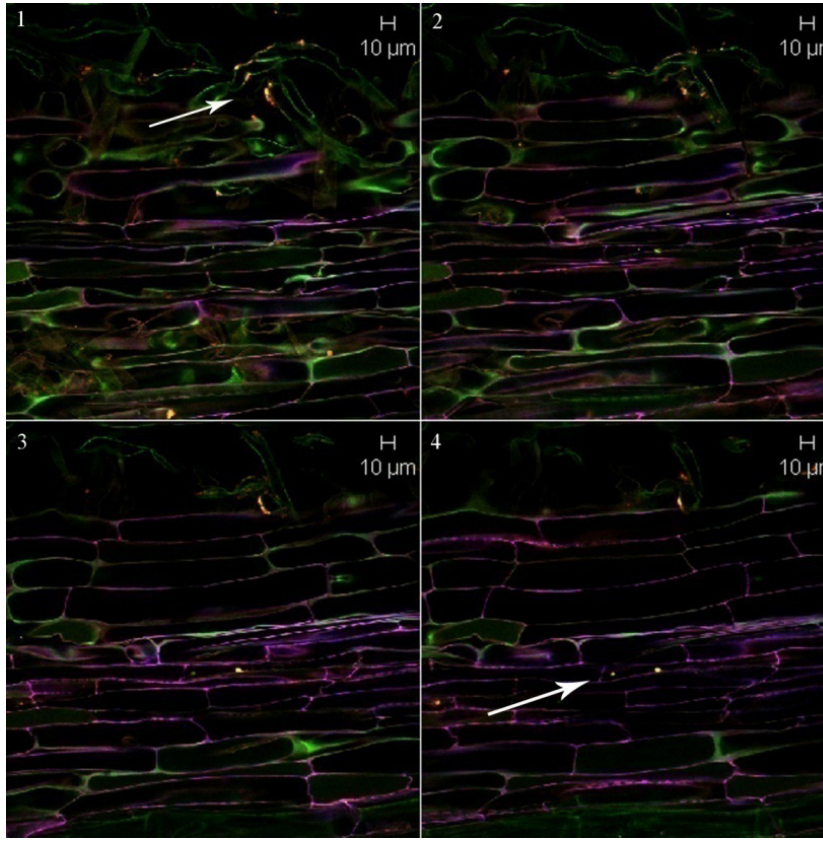

Figure 7: CLSM micrograph of endophyt colonization root by *Salmonella typhimurium*.

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