A one-step PCR assay for the detection of pathogenic
*Y. enterocolitica* in artificially contaminated fecal samples
and lymphoid tissue

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Abstract: In order to specifically detect pathogenic, plasmid bearing *Yersinia
enterocolitica*, we have developed a polymerase chain reaction (PCR) assay based
on the plasmid located gene *yopT*. A substantial number of mismatches within the
*yopT* coding sequence between *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y.
pestis* was used to generate a primer pair that exclusively detects pathogenic *Y.
enterocolitica* with a high sensitivity and specificity. When this PCR assay was
used for the detection of pathogenic *Y. enterocolitica* cells in artificially inoculated
fecal samples and lymphoid tissue of pigs, levels as low as $10^2$ cells per gram feces
and $10^4$ cells per gram lymphoid tissue could be detected if an 24 h pre-enrichment
in Luria Bertani-Bouillon was performed prior to the PCR.

Keywords: *Yersinia* – *yopT* – PCR – pre-enrichment - diagnostic

Introduction: Foodborne *Yersinia enterocolitica* are a major cause of human
gastroenteritis. Pigs, inapparently infected with *Y. enterocolitica* are considered to
be the main reservoir for human infections. *Yersinia* pathogenesis is a
multifactorial process dependent upon the expression of genes located both, on a
virulence plasmid (pYV) of 70 to 75 kbp and on the bacterial chromosome.
However, virulence is highly associated with the presence of the *Yersinia* virulence
plasmid (Cornelis et al., 1998).

Most phenotypic markers for differentiating pathogenic from non-pathogenic
*Yersinia* are time-consuming and sometimes inconsistent (Neubauer et al., 2001).
Accordingly, development of rapid methods for the detection of pathogenic *Y.
enterocolitica* is important. The purpose of this study was to develop a rapid,
sensitive and specific one step PCR assay based on the plasmid-located virulence
gene *yopT* to detect plasmid harboring *Y. enterocolitica* in fecal samples and
lymphoid tissue.

Materials and Methods:
Oligonucleotide primers and PCR conditions. The developed PCR assay is based
on the plasmid located gene *yopT*. Computer assisted (ClustalW 1.8) nucleotide
sequence comparison of yopT from Y. enterocolitica, Y. pseudotuberculosis and Y. pestis revealed a substantial number of mismatches within the yopT coding sequence between the three species. This finding was used for the design of pathogenic Y. enterocolitica specific oligonucleotides. The primer pair yopT-fw1 (5'-TATGTGCACATTGGATT TT-3') and yopT-r1 (5'-AATGATACATAGAATT TT-3') was used to amplify a 478 bp fragment of yopT (Arnold et al., 2001). For template preparation single colonies were suspended in 500 μl double deionized water (ddH2O). The mixture was heated for 15 min at 94 °C. After centrifugation, 10 μl of the supernatant were used as template in the PCR assay and carried out in a final volume of 50 μl. Optimization of cycling conditions resulted in initial denaturation for 5 min at 94 °C followed by 35 cycles each consisting of 1 min denaturation at 94 °C, 40 sec annealing at 43 °C, and 1 min extension at 72 °C, and a final extension step of 7 min.

Inoculation of fecal samples and lymphoid tissue. Three grams of feces and lymphoid tissue (Tonsils, Mandibular ln.) were each mixed with 3 ml of a bacterial suspension of Y. enterocolitica Y11 (DSM 13030) containing 3 x 10^8 to 3 x 10^9 cells and homogenized using a Stomacher 400 at high speed for 2 min. 1 ml of this mixture was used for enrichment in 9 ml of non-selective Luria Bertani-Bouillon (LB). After 24 h of enrichment, 1 ml of the suspension was centrifuged at 300 x g for 3 min in an Eppendorf tube to remove crude particles. The supernatant was centrifuged again at 10,000 x g for 10 min. The pellet was washed twice in sterile PBS, resolved in a final volume of 200 μl lysis buffer, incubated for 1 h at 56 °C and heated to 95 °C for 10 min to inactivate the proteinaseK in the lysis buffer. 10 μl of the supernatant was subjected to PCR analysis. For direct bacterial DNA-extraction from artificially contaminated feces without foregoing enrichment the new QIAamp DNA Stool Kit® was tested. Here, direct DNA-extraction from artificially contaminated lymphoid tissue is carried out with several centrifugation steps.

Results: In order to prove the specificity of the PCR assay, various pathogenic and non-pathogenic strains of Y. enterocolitica and Y. pseudotuberculosis of different serovar/biovar combinations were investigated. Y. pestis and members of other bacteria causing similar infections in humans and animals were included. The specific PCR product of 478 bp was exclusively amplified from DNA of plasmid bearing Y. enterocolitica. No false-positive results from DNA of other bacteria were obtained. Therefore, this assay is highly specific for plasmid bearing Y. enterocolitica. To examine the detection limit of the PCR assay, a series of 10-fold dilutions of Y. enterocolitica-suspension beginning with 10^8 cells/ml in ddH2O was carried out. Cells from each dilution were suspended in lysis buffer and incubated for 1 h at 56 °C. Using these conditions 10^2 cells were sufficient to give a positive PCR reaction. In artificially spiked stool samples and lymphoid tissue Yersinia was detectable with an initial inoculum of 10^2 cells/g and 10^1 cells/g after 24 h of
enrichment in LB. The detection limit of the QIAamp DNA Stool Kit was 10^6 cells/g for feces. By the centrifugation method, at least 10^6 cells/g lymphoid tissue were necessary for a positive PCR reaction.

**Discussion:** We could show that our PCR assay is highly specific and sensitive for pathogenic *Y. enterocolitica* in a single PCR reaction. An identical specificity and sensitivity was obtained with pure cultures, in pre-enriched fecal samples and lymphoid tissue. Therefore, this assay is suitable to detect pathogenic *Yersinia* in different environments. Rapid detection and discrimination of enteropathogenic strains of *Y. enterocolitica* from other *Yersinia* strains as well as from pathogenic members of the genus *Enterobacteriaceae* is important for diagnostic purposes and for the hygiene control in food production. The combination of the developed PCR assay with a 24 h pre-enrichment step in LB might be a useful tool to detect pathogenic *Y. enterocolitica* in fecal samples and lymphoid tissue in routine diagnostics. The QIAamp DNA Stool Kit and the centrifugation method, however, gave only unsatisfying results. Therefore, it can be assumed that the application of the Qiagen kit is limited and might have been optimized to detect pathogens in human faecal samples.

In conclusion, the PCR system described in this report is highly specific for the detection of pathogenic *Y. enterocolitica* in fecal samples and lymphoid tissue within 28 h (24 h of enrichment). Thus, this PCR system is a useful and inexpensive tool for rapid detection of pathogenic *Y. enterocolitica*. Future investigations will prove whether the developed assay is also suitable to rapidly detect pathogenic *Y. enterocolitica* in experimentally infected pigs.

**Acknowledgements / Financiers:**
This work was supported by the Deutsche Forschungsgemeinschaft (DFG) under the project GRK-39/2.

**References:**
