DEVELOPING REAL-TIME PCR ASSAY FOR DETECTION OF MULTI-DRUG SALMONELLA STRAINS IN PIGS

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Abstract The main objective of this study was to develop a rapid, sensitive and accurate real-time detection assay for multi-drug resistant (MDR) Salmonella strains isolated from pigs. Initially, standardized procedures for use with real-time PCR using SYBR® green were developed to evaluate selected primers, detection limitations using two predominant strains: S. Typhimurium phage types DT104 and DT193. The use of bacterial lysate and purified DNA samples was also compared. Lysate dilutions for concentrations (100-10^-5) resulted in widely varied amplification as reflected in amplification curves. In contrast, purified DNA samples diluted to 10^-8 µg/mL resulted in lower threshold cycles thus was found to be a more reliable procedure. The low detection limit and therefore, increased sensitivity of real-time PCR suggests this technology could be the cornerstone for development of assays for potential use on farms, in slaughter facilities, and by veterinarians in field efforts to detect pathogens in biological samples before organisms cause food-borne illness in humans.

Introduction Human salmonellosis cases caused by non-typhoid strains of Salmonella are projected to cause 600 deaths and cost $2.8 billion dollars annually within the United States (www.ers.usda.gov). The ubiquitous nature of Salmonella enterica within swine production and pork processing facilities may create a reservoir for isolates that are multi-drug resistant thus posing a potential risk to human health (Gebreyes et al., 2004; Martin et al., 2003). Such isolates include serovar Typhimurium and phage types DT104 and DT193 which are resistant to ampicillin, streptomycin, tetracycline, sulfamethoxazole, and chloramphenicol or kanamycin, respectively (Gebreyes and Altier, 2002). Therefore, utilization of technology and the development of methods that rapidly and accurately detect multi-drug resistant (MDR) Salmonella is of paramount importance in the effort to prevent food-borne illness.

Molecular methods including real-time PCR are frequently used to diagnose, characterize, and track pathogens causing food-borne disease (Gebreyes, 2003). Real-time PCR is a rapid, quantitative method for measuring the kinetics of target sequence amplification by threshold cycles and specificity of the amplification by melt curves (www.appliedbiosystems.com). Daum, et al. (2002) showed that real time PCR technology required only 3 hours to accurately diagnose an outbreak of salmonellosis due to consumption of contaminated chicken. Additional sensitivity of this technology can be obtained by using probes like molecular beacons that bind exclusively to the target rather than intercalating dyes such as SYBR® green that bind to all dsDNA (Gebreyes, 2003). The use of SYBR® green necessitates rigorously optimized procedures to minimize amplification of non-specific targets (www.appliedbiosystems.com).

Notably when inter-laboratory real-time PCR procedures are standardized regardless of fluorophore type, the capability of detecting Salmonella from swine and poultry carcass samples produced specific results with 97.5% accuracy (Malorny et al., 2003). Accuracy of detection can also vary by sample preparation and DNA extraction procedures used in conjunction with real time PCR (De Medici et al., 2003). In light of these stipulations, only methods producing reproducible results with accurate melting temperatures should be considered for application in a diagnostic protocol designed to ensure public health.

Materials and Methods Two MDR Salmonella enterica serovar Typhimurium isolates (DT104 and DT193) were used for standardization. Eight other Salmonella strain and phage types were used to test the robustness of the procedure in preparation for additional studies in this area. These isolates were streaked onto LB agar and incubated at 37°C for 24 h. Single colonies were used to inoculate tubes containing LB broth for enrichment.

Two boiling lysis and one DNA purification method was used to isolate enriched Salmonella samples from LB broth cultures. First, the boiling lysis method was used to prepare Salmonella Typhimurium factor-10 serial dilutions to 10^4 and 10^5 µg/mL. Aliquots were centrifuged for 10min
at 10000 x g and 4°C and supernatant discarded by aspiration. The pellet was resuspended in 300uL TE buffer and incubated for 15min at 100°C on a heating block. Aliquots were immediately transferred to chill on ice for 10 min and then centrifuged for 5min at 14000 x g and 4°C. All supernatants from both boiling methods were carefully transferred to a 1.5mL microcentrifuge tube. An additional incubation step was added in the second boiling method as outlined by Malorny, et al. (2003). All sample aliquots regardless of boiling method were stored at -20°C. DNA extractions were subsequently performed for comparison. DNA from Salmonella enterica serovars was purified using the Qiagen® DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s handbook guidelines. The final purified product was serially diluted by factor-5 and factor-10 to 10^-8 µg/mL and stored at 4°C.

Primers previously designed using Beacon Designer 3 by Premier Biosoft were used to amplify resistance regions unique to DT104 and DT193 phage types. Four primers, two for each phage, were used to detect, \( \text{bla}^\text{Tem} \) and \( \text{AphA} \) for DT193 and \( \text{bla}^\text{Pse1} \) and \( \text{CmlA} \) for DT104 resistance alleles. Standardization of pse1 and tem primers was tested at 0, 40, 80, 100, 250, 350, 400, and 500 nM concentrations. Amplification of targets was optimized by a primer concentration of 350nM.

All samples were amplified in a 96 micro-well plate using the iCycler iQ real-time PCR machine manufactured by Bio-Rad Laboratories, Inc. Standardization of total reaction volumes was performed using 50uL volumes as recommended by Bio-Rad Laboratories, Inc. and 25uL volumes. All subsequent runs were performed using a 25uL total reaction volumes. Targets were amplified from 1, 5, and 10µL concentrations of lysed cells and 1uL purified DNA aliquots. PCR program protocols generated amplification and melt curves for determination of sensitivity and specificity target values.

Results Total reaction volumes of 25µL and 50µL resulted in target amplification above threshold after 10 to 15 cycles. Since this result occurred during the standardization process, the lesser, 25µL, volume was used in all subsequent runs. The 350nM primer concentration specifically detected and amplified their respective resistance genes.

Serial dilutions of S. Typhimurium lysates resulted in non-specific or no amplification of targets for 1, 5, and 10µL sample volumes. Lysate samples did not produce consistent amplification beyond one factor-10 dilution. Serial dilutions of 53µg/mL purified DNA resulted in specific amplification of targets. The number of amplification cycles increased as the sample concentration decreased which is illustrated by the standard curve (Figure 1). Standard curves are reported to

![Figure 1: Amplification curve for S. Typhimurium phage type DT104 factor-10 serial dilutions purified DNA from 100 to 10-8 µg/mL showing threshold cycle as it relates to sample concentration.](image-url)
estimate the number of molecules in a range of ±6-21% precision (Rutledge and Côté, 2003). Based on this data derived from amplification and standard curves, subsequent amplification of samples was considered relevant at or above the cycle predicted by the standard curve for that specific concentration as verified by the melt curve. After analysis of these curves, the 3 fold factor-10 serial dilution of the sample was used for all subsequent amplification runs.

Other Salmonella strain isolates including Derby (IE1) and Heidelberg (MM258) at a concentration of 0.053µg/mL were amplified by DT104 or DT193-specific primers. Relevant amplification of targets at this concentration was considered to occur at or above 31 cycles. Amplification may be due to non-specific binding of primers to sequences that are not involved with resistance in Derby or Heidelberg. Additional profiling including disk diffusion must be done on the Derby (IE1 and ID5) and Heidelberg (MM258) isolates to determine resistance patterns.

Discussion A total reaction volume of 25µL produces amplification of targets comparable to the manufacture suggested 50µL total reaction volume. The decreased volumes of primers and SYBR® green may reduce waste and cost associated with amplification of targets. In an effort to further minimize cost and sample preparation time, specificity and sensitivity of boiling lysis procedures was assessed. Unfortunately in this study, cell lysis methods proved inadequate for reliable amplification of targets. After modifying the lysis procedure and further analyzing the melt curves, we determined extraction of DNA was best carried out using Qiagen® DNeasy Tissue kits.

Using purified DNA from DT104 and DT193, dynamic range analysis of standard curves appeared accurate for predicting target cycle amplification as a function of sample concentration. Therefore, detection limitations were determined to exist for purified DNA samples at concentrations less than 10-4 µg/mL. Based on preliminary runs, tem and pse1 primers showed promise for use with non-Typhimurium and non-DT104/DTD193 isolates. By increasing the number of samples analyzed as well as definitively determining resistance phenotypes of Heidelberg and Derby isolates, robustness of this technology in the field can be accurately determined.

Conclusions This procedure is being refined to make it applicable in the field. The use of these primers across serovars and phage types may aid in epidemiologic tracking of antimicrobial resistance patterns accumulating in commercial swine production facilities.

References


