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Development and Standardization of Multiplex qPCR for the Detection of Salmonella Enteritidis, Typhimurium, Gallinarum, Pullorum, and Heidelberg Serovars in the Poultry Industry

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Brazil was the second largest poultry producer in 2022, so poultry farming plays a crucial role in Brazilian economy. However, it faces challenges due to Salmonella, which is present in over 35% of poultry products and is responsible for recurring foodborne outbreaks. Serotyping Salmonella serovars is essential for understanding salmonellosis, and multiplex qPCR provides a faster and more accurate diagnosis than microbiological culture. The aim of this study was to standardize multiplex qPCR for the simultaneous differentiation of the serovars Enteritidis, Typhimurium, Gallinarum, and Pullorum, which are recommended for monitoring by Brazilian legislation, as well as the Heidelberg serovar, which has been frequently found in poultry production. The samples used in the study were sourced from scientific research projects and collections from the routine of the Laboratory of Food Sanitary Inspection at the Serviço de Orientação a Alimentação Publica (SOAP) and the Ornithopathology Service, both from FMVZ (UNESP – Botucatu). The DNA from Salmonella samples was extracted using an in-house adapted protocol with magnetic beads. Confirmation that all samples belonged to the Salmonella genus was performed by qPCR, targeting a conserved region of the invA gene. To standardize multiplex qPCR for the serovars Enteritidis, Typhimurium, Pullorum, and Gallinarum, primers and probes previously selected based on studies identifying specific regions for each serovar were used. The probe specific for S. Heidelberg was designed using the Geneious software, employing reference genomes from the National Center for Biotechnology Information (NCBI) and primers described in previous studies, with consideration of the fluorophores to be used. After selecting the primers and probes for the multiplex qPCR, the reagent concentrations were standardized. Three mixes were prepared with different concentrations of probes and primers for S. Heidelberg, while keeping the concentrations of nuclease-free water, GoTaq® Probe qPCR Master Mix, and the primers and probes for Enteritidis, Typhimurium, Gallinarum, and Pullorum constant, according to previous standardizations. The detection threshold for the proposed multiplex qPCR was determined by serial dilutions (1:10 to 1:10,000,000). The sensitivity and specificity of the multiplex qPCR were then validated. After extraction and detection of the invA gene by qPCR, 21 S. Heidelberg strains were obtained. qPCR with SYBR Green, using specific primers for S. Heidelberg, demonstrated high specificity. The probe designed for S. Heidelberg efficiently annealed in the third mix (0.2 µM SH-F, 0.2 µM SH-R, and 0.1 µM SH-PROBE), resulting in slightly lower Cq values compared to the other mixes. The detection threshold was achieved up to the fifth serial dilution, with a maximum Cq of 35.82. The standardized multiplex qPCR showed high sensitivity and specificity for the 21 S. Heidelberg strains. This study demonstrates that multiplex qPCR is a highly effective and specific tool for detecting S. Heidelberg

and other Salmonella serovars, providing a sensitive and precise approach for monitoring and controlling in food safety.

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