Establishment and Application of a duplex real-time qPCR method for detection of *Salmonella* spp. and *Serratia fonticola* in imported feedstuffs

Running title: RT-qPCR analysis of feedstuff

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Abstract *Salmonella* spp. is a high-risk bacterial pathogen that is monitored in imported animal-derived feedstuffs. *Serratia fonticola* is the bacterial species most frequently confused with *Salmonella* spp. in traditional identification methods based on biochemical characteristics, which are time-consuming and labor-intensive, and thus unsuitable for daily inspection and quarantine work. In this study, we established a duplex real-time qPCR method with *invA*- and *gyrB*-specific primers and probes corresponding to *Salmonella* spp. and *S. fonticola*. The method could simultaneously detect both pathogens in imported feedstuffs, with a minimum limit of detection for *Salmonella* spp. and *S. fonticola* of 197 copies/µL and 145 copies/µL, respectively (correlation coefficient $R^2 = 0.999$ in both cases). The amplification efficiency for *Salmonella* spp. and *S. fonticola* was 98.346% and 96.49%, respectively. Detection of clinical samples was consistent with method GB/T 13091-2002, and all 20 artificially contaminated imported feed samples were positively identified. Thus, the developed duplex real-time qPCR assay displays high specificity and sensitivity, and can be used for the rapid and accurate detection of genomic DNA from *Salmonella* spp. and *S.*

42   *fonticola* within hours. This represents a significant improvement in the efficiency of
43   detection of both pathogens in imported feedstuffs.

44

45   **Importance** Imported feedstuffs must be tested for pathogenic *Salmonella* species
46   that represent a biological hazard. Various non-*Salmonella* colony-forming
47   species belong to *Enterobacteriaceae*, and *Serratia fonticola* forms colonies of similar
48   color and morphology to *Salmonella* spp., leading to confusion in daily quarantine
49   tests. Traditional methods based on biochemical and serological characteristics are
50   cumbersome and labor-intensive, and unable to fully support current quarantine
51   testing demands. Thus, there is an urgent need to develop a rapid and accurate method
52   for the effective identification of these pathogens. The duplex real-time qPCR method
53   established herein can rapidly identify *Salmonella* spp. and *S. fonticola*, and has great
54   potential for application to feed safety and prevention of exterior pathogens.

55

58

Introduction

60   *Salmonella* spp. are ubiquitous Gram-negative bacteria in the environment and
61   include six different subspecies and more than 2000 serotypes that infect a wide range
62   of hosts, often causing severe food poisoning outbreaks in humans and other animals.
63   People infected with *Salmonella* can develop diarrhea, fever, and suffer dehydration,
64   hence *Salmonella* spp. are of significance to public health.

65

66   *Serratia fonticola* is a species belonging to the *Serratia* genus that was first isolated
67   from water and soil in 1979 (1). Subsequent studies showed that *S. fonticola*, a
68   member the Gram-negative *Enterobacteriaceae* family that includes *Salmonella* spp.,
69   is also ubiquitous in environments such as water, soil, plants, and the gastrointestinal
70   tract of humans and other animals (2). Research has revealed that *S. fonticola* can
71   infect various tissues and organs in humans (3, 4), causing septic arthritis (5),
72   septicemia (6, 7), gastrointestinal tract infections (8, 9), and surgical infections (10).
73   Therefore *S. fonticola* have been defined as an important opportunistic pathogen.

74

75   At present, the detection of *Salmonella* spp. in imported animal-derived feeds (fish
76   meal and chicken powder) involves non-selective enrichment, selective enrichment,
77   selective platelet culturing, biochemical culturing of suspected *Salmonella* colonies
78   (triglyceride tests, etc.), and even serological typing. These conventional methods
79   require at least 3 days to detect *Salmonella* spp. in daily quarantine work. Furthermore,
80   suspected *Salmonella* colonies based on selective plate isolation and culturing of
81   imported animal-derived feeds often turn out to be S. *fonticola* when biochemical
82   properties are investigated (11). The enzyme linked immune sorbent assay (ELISA)
83   method is commonly used for detecting pathogens in imported animal-derived
84   feeds, but this method can produce incorrect results due to its cumbersome
85   operational steps. A novel method involving polymer fluorescent nanoparticles

86　as biosensor probes has been described for the detection of *Salmonella* (12), which
87　takes only 3 h, but cannot be applied to large volume detection due to its prohibitively
88　high cost.

89

90　PCR is widely used in the detection of pathogens due to its high sensitivity and
91　specificity, and results can often be obtained within several hours. Numerous
92　traditional PCR and multiplex PCR methods have been developed for the detection of
93　species belonging to both the *Salmonella* and *Serratia* genera (13-17). Multi-PCR
94　approaches can differentiate two or more pathogens in one amplification, however,
95　products are easily contaminated during agarose gel electrophoresis, which increases
96　false-positive and false-negative results. Thus, a more reliable approach such as
97　real-time fluorescence quantitative PCR (RT-qPCR) would be more desirable for the
98　rapid and accurate detection of pathogens. RT-qPCR methods are known to be fast,
99　reliable, and highly efficient. Several single RT-qPCR methods for detecting *S.*
100　*nematodiphila* (18), *S. marcescens* (19-21), and *Salmonella* spp. (22-24) have been
101　described, and multiplex RT-qPCR detection methods have also been reported
102　(25-28).

103

104　Previous studies on fish meal and other animal-derived feeds have mainly focused on
105　the detection and epidemiology of *Salmonella*, *Shigella*, *Escherichia coli*, and other
106　common pathogenic bacteria. A method for the simultaneous detection of *Salmonella*
107　spp. and *S. fonticola* in imported animal-derived feeds using RT-qPCR has not been
108　reported. Such a method could provide rapid differential diagnosis of suspected
109　*Salmonella* colonies after selective plate separation and culturing. This would
110　undoubtedly improve the detection of *Salmonella*, and greatly shorten the time
111　required for subsequent biochemical and serological identification, saving valuable
112　manpower and material resources.

113

114　The duplex real-time qPCR method established in the present work provides a useful
115　tool for the simultaneous detection of *Salmonella* spp. and *S. fonticola*. The method
116　has important theoretical significance and great potential for improving the safety of
117　imported feeds by rapidly identifying bacterial pathogens and facilitating effective
118　quarantining in a more timely manner than traditional detection methods.

119

120　**Materials and Methods**
121　**Bacterial strains.** A total of 48 tested strains were used in this study, including 17
122　reference strains from six different collection centers, and 31 isolates from imported
123　fishmeal (Table 1, 2, and 3). All experimental strains were streaked on nutrient agar
124　plates and cultured in Luria-Bertani (LB) broth at 37°C overnight (~18–24 h), except
125　for *S. marcescens*, which was grown at 23°C for 24 h. An established single colony
126　was inoculated into 3 mL of LB broth for 8 h and the resultant culture was harvested
127　for genomic DNA extraction.

128

129　**Sample collection and bacterial isolation.** A 25 g sample of fishmeal was added to

3

130   225 mL of buffered peptone and incubated at 36 ± 1°C for between 16–20 h. A 10 mL
131   sample of this pre-enrichment culture was transferred into 100 mL of enrichment
132   solution containing selenite cystine, cultured at 36 ± 1°C for 24 h, and inoculated onto
133   selective medium designed for *Salmonella* spp. Two *Salmonella* spp. colonies were
134   cultured at 36 ± 1°C for 48 h in selective medium, one on the medium slope, the other
135   punctured through the agar, and both were then inoculated on trisaccharide iron
136   medium. Meanwhile, suspicious colonies were inoculated onto lysine decarboxylase
137   medium and cultured at 36 ± 1°C for 18–24 h (or up to 48 h if necessary). Colonies
138   from positive samples presumed to be *Salmonella* spp. were further identified using a
139   VITEK II Compact 30 instrument (bioMérieux Technologies Inc., SA, French) with a
140   GN card according to the manufacturer's specifications.

141

142   **Species-specific primer and probe design.** The *invA* sequences of *Salmonella* spp.
143   were aligned to identify conserved and specific regions using CLUSTAL W software
144   (29). A series of sense and antisense primers were designed based on these conserved
145   and specific regions using ABI ViiA7 PrimerExpress software (Life Technologies Inc.,
146   Foster City, CA, USA), and the final specific primer pair and dual-labelled probe
147   (Table 4) targeting the *Salmonella* spp. virulence gene were determined using
148   the basic logical alignment search tool (BLAST) (30). Primers and dual-labelled
149   probe targeting the *gyrB* gene were as described previously (31). Two primers and
150   probe sets were synthesized by Sangon Biotech (Sangon Biotech Co., Ltd. Shanghai,
151   China), and probes labeled with the fluorescent reporter dye
152   carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) targeting *invA* and
153   6-carboxyfluorescein (FAM) targeting *gyrB* were covalently coupled to the 5′-end,
154   with Black Hole Quencher 1 (BHQ-1) at the 3′-end.

155

156   **Extraction of bacterial genomic DNA.** DNA extraction from isolates and reference
157   strains was performed with a TIANamp Bacteria DNA Kit (Tiangen Biotech, Co., Ltd,
158   Beijing, China) according to the manufacturer's recommendations. DNA was eluted
159   with sterile water and stored at -20°C until use. DNA purity and concentration were
160   determined using a Quawell Q6000+ spectrophotometer (Quawell Technology, Inc.,
161   USA).

162

163   **Screening of the optimum annealing temperature of primers.** The 20 μL qPCRs
164   contained 10 μL of (2×) Premix Ex Taq (TaKaRa Biomedical Technology Co., Ltd,
165   Beijing, China), 0.4 μL of each primer (10 μM) and probe (10 μM), 1 μL of bacterial
166   DNA, and 7.8 μL of Rnase-free ddH$_2$O. Reactions were performed on a Bio-rad
167   CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc., USA) using a two-step
168   method. Thermal cycling conditions consisted of an initial denaturation at 95°C for 30
169   s, followed by 40 cycles at 95°C for 5 s and 65.0, 64.5, 63.3, 61.4, 59.0, 57.0, 55.7, or
170   55.0°C (triplicates for each temperature) for 34 s to determine the optimal annealing
171   temperature of the two primer sets. CFX Manager Software (Bio-Rad Laboratories,
172   Inc., USA) was employed to monitor PCR amplification, and the collect and analyze
173   amplification data.

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174

175 **Single real-time qPCR assays.** Two simplex real-time qPCR detection methods for
176 the detection of *Salmonella* spp. and *S. fonticola* were established using primers and
177 probes targeting *invA* and *gyrB* genes, respectively, using an Applied Biosystems
178 ViiA 7 real-time PCR system (Life Technologies Inc., Foster City, CA, USA).
179 Reaction conditions were determined after various PCR parameters were tested
180 according to the information supplied with the reagents. The optimized 20 μL PCR
181 contained 10 μL of 2× Premix Ex Taq, 0.4 μL of each primer (10 μM) and probe (10
182 μM), 0.2 μL of ROX Reference Dye Ⅱ (50×), 1 μL of bacterial DNA, and 7.6 μL of
183 Rnase-free ddH$_2$O. Optimized thermal cycling conditions involved an initial
184 denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 64°C for 34 s.
185 ViiA 7 Software (Life Technologies Inc., Foster City, CA, USA) was employed to
186 monitor PCR amplification, and the collect and analyze amplification data.

187

188 **Specificity of simplex real-time qPCR assays.** To verify the specificity of the two
189 simplex real-time qPCR assays, tests were performed by amplifying genomic DNA
190 extracted from strains listed in Table 1, 2, and 3. Genomic DNA was extracted from
191 29 *Salmonella* spp. and 19 non-*Salmonella* species and used to determine the
192 specificity of the designed primers and probes targeting the *invA* gene. The non-*S.
193 fonticola* control containing 44 non-*S. fonticola* organisms, comprising eight different
194 species belonging to the genus *Serratia*, and 36 *Enterobacteriaceae* strains, was used
195 in RT-qPCR to confirm cross-reaction of *gyrB* gene-specific PCR primers and probes.

196

197 **Construction of recombinant plasmids.** PCR products amplified with primers
198 targeting *invA* and *gyrB* genes were purified using a DNA Fragment Purification Kit
199 (Tiangen Biotech, Co., Ltd) after agarose gel electrophoresis, and sequenced by
200 Sangon Biotech to confirm the identity of the target genes used in this study. The
201 pMD19-T vector (Sangon Biotech) was used for cloning target genomic sequences,
202 and the resultant constructs were transformed into competent *Escherichia coli* DH5α
203 cells. Recombinant plasmids carrying each target gene were confirmed, and
204 subsequent DNA sequencing was also performed by Sangon Biotech. Amplified
205 sequences were compared with original sequences deposited in the GenBank database
206 using NCBI BLAST.

207

208 **Sensitivity of single real-time qPCR assays.** The purity and concentration of
209 recombined plasmids pMD-*invA* and pMD-*gyrB* were determined using a Quawell
210 Q6000+ spectrophotometer. Plasmids were 10-fold serially diluted nine times and
211 subjected to real-time qPCR to construct standard curves (three technical replicates
212 for each dilution), from which we determined both the amplification efficiency and
213 the minimum detection limit of the two real-time qPCR methods. The plasmid copy
214 numbers was calculated using the following formula (32):

215

216 Copy number = (DNA amount (ng) $\times$ 6.022$\times10^{23}$ / length (bp) $\times 10^9 \times$ 650).

217

5

218   **Duplex real-time qPCR assay.** A duplex real-time qPCR assay was established based
219   on the two simplex RT-qPCR assays developed for the detection of *Salmonella* spp.
220   and *S. fonticola* using an equal amount of genomic DNA from the two pathogens in
221   the same reaction. Optimal PCR conditions were ultimately determined based on the
222   simplex RT-qPCR parameters described above by varying a single factor while all
223   other parameters remained constant. The main factor to be investigated was the
224   concentration of the two primer and probe sets since the annealing temperatures of
225   primers and probes was already calculated.

226

227   **Reproducibility and stability tests.** To analyze the reproducibility and stability of
228   the established duplex real-time qPCR method, recombinant pMD-*invA* and
229   pMD-*gyrB* plasmids were 10-fold serially diluted five times (each dilution was tested
230   in triplicate). A total of 96 reactions were simultaneously performed using the *invA*-
231   and *gyrB* recombinant plasmids in the same real-time qPCR mixture to verify
232   reproducibility. The coefficient of variation (CV) based on quantification cycle (Cq)
233   values for each test was used to evaluate the performance of this approach.

234

235   **Detection of artificially contaminated imported feedstuffs.** We tested 20 imported
236   fishmeal samples not contaminated by either *Salmonella* spp. or *S. fonticola*. A 25 g
237   portion of 10 of the fishmeal samples was artificially contaminated with *Salmonella*
238   spp., while the other 10 samples were contaminated with *S. fonticola*, and
239   contaminated samples were added to 225 mL of buffered peptone enrichment solution
240   and incubated at 36 ± 1°C for 16–20 h. A 1 mL sample of the pre-enrichment culture
241   was transferred into 10 mL of enrichment solution containing selenite cystine,
242   cultured at 36 ± 1°C for 24 h, and 1 mL of the resultant culture was used for genomic
243   DNA extraction using the TIANamp Bacteria DNA Kit (Tiangen Biotech, Co., Ltd).
244   Finally, RT-qPCR was performed for the detection of *Salmonella* spp. and *S. fonticola*,
245   and the national standard method (GB/T13091-2002) was also employed for
246   verification.

247

248   **Detection of clinical imported feed samples.** Ten imported fishmeal samples
249   infected with *Salmonella* spp. and four imported fishmeal samples infected with *S.*
250   *fonticola* were collected during quarantine work between June 2015 and June 2017.
251   Genomic DNA was extracted from each sample after treatment as described above,
252   real-time qPCR was performed for detection of these two pathogens, and the national
253   standard method (GB/T13091-2002) was also employed for verification.

254

255   **Results**
256   **Determination of optimum annealing temperature.** Genomic DNA from
257   *Salmonella enteritidis* and *S. fonticola* was used as a template to determine optimal
258   annealing temperatures for the two primer sets by testing between 55 and 65°C using
259   a Bio-Rad fluorescence quantitative PCR instrument. RT-qPCR using the designed
260   primers and probes yielded the highest fluorescence intensity of amplified products at
261   am annealing temperature of 64.5°C for both *invA*- and *gyrB*-specific primers and

262   probes, and this annealing temperature was employed in subsequent experiments.

263

264   **Specificity of single real-time qPCR assays.** The specificity of the two single
265   real-time qPCR assays was verified by amplifying genomic DNA extracted from
266   reference strains and laboratory isolates. As shown in Figure 1, the real-time qPCR
267   assay detected *Salmonella* spp. only, while no fluorescent signal was observed for any
268   non-*Salmonella* spp. strains or blank controls. However, the real-time qPCR assay
269   targeting *S. fonticola* generated four specific amplification curves for the detection of
270   four *S. fonticola* strains, while non-*S. fonticola* and blank controls were not amplified
271   (Figure 2). Thus, the specificity of the two real-time qPCR assays was 100%, with no
272   detectable fluorescent signal for negative samples or blank controls.

273

274   **Standard curves and sensitivity of single real-time qPCR assays.** Recombinant
275   *invA*- and *gyrB*-containing plasmids were 10-fold serially diluted nine times, resulting
276   in real-time qPCR amplicon copy numbers from 1. 97×10^{10} copies/μL to 1. 97×10^{2}
277   copies/μL, and 1.45×10^{10} copies/μL to 1.45×10^{2} copies/μL, respectively. The standard
278   curve for *invA* has a γ intercept of 48.116, a slope of -3.267, and a mean efficiency of
279   102.344% (Figure 3). The *gyrB* standard curve has a γ intercept of 42.919, a slope of
280   -3.242, and a mean efficiency of 103.429% (Figure 4). As shown in Fig. 5 and Fig. 6,
281   the single quantitative real-time PCR assays could detect *Salmonella* spp. and *S.*
282   *fonticola* at concentrations as low as 197 and 145 copies per reaction, respectively.

283

284   **Establishment of the duplex real-time qPCR assay.** Figure 7 and Figure 8 shows
285   amplification plots and standard curves for the duplex real-time qPCR assay
286   established for simultaneous detection of *Salmonella spp.* and *S. fonticola* developed
287   with recombinant pUCm-*invA* and pUCm-*gyrB* plasmids. Standard curve slopes are
288   -3.362 and -3.409 for the detection of *invA* and *gyrB*, respectively, indicating an
289   amplification efficiency of 98.346% and 96.49%. A correlation coefficient
290   consistently higher than 0.999 indicates effective simultaneous detection of two kinds
291   of pathogens in one real-time qPCR assay without cross-reaction(data shown in
292   Table5).

293

294   **Testing of reproducibility and stability.** Five recombinant *invA* and *gyrB* plasmid
295   dilutions were simultaneously used as substrates to evaluate the reproducibility and
296   stability of the assay. As shown in Table 6, the standard deviation was no more than
297   0.153 for all reactions, and the coefficient of variation was less than 0.73%.
298   Additionally, Ct values for the same plasmid samples obtained from same
299   experiments were determined to analyze the performance of the developed real-time
300   qPCR method. The results showed that amplification plots for all 96 replicate
301   reactions were almost coincident in the vicinity of the threshold line (data not shown).
302   Thus, the established quantitative real-time PCR assay has high stability and
303   reproducibility.

304

305   **Duplex real-time qPCR analysis of imported feedstuffs.** Imported feedstuffs not

306 infected with either *Salmonella* spp. or *S. fonticola* were artificially contaminated with
307 the corresponding pathogens. Half of the feed samples were contained with
308 *Salmonella* spp., while the other half were contained with *S. fonticola*, and genomic
309 DNA was extracted for analysis after treatment. Detectable fluorescent signals were
310 observed for all artificially contaminated feed samples following amplification.
311 Furthermore, analysis of clinical imported fishmeal samples resulted in 10
312 amplification plots for the detection of *Salmonella* spp. and four amplification plots
313 for the detection of *S. fonticola* (data not shown). Finally, the results of the detection
314 of artificial and clinical samples were consistent with the national standard method
315 (GB/T13091-2002) that was also employed for verification.

316

317 **Discussion**
318 Many target genes for the detection of genus *Salmonella* species have been reported,
319 including *afgA*, *hilA*, *spvC*, *sef* (33, 34), *fliC*, *fliB*, *iroB*, *rfbJ* (35), *ompC* (36), *spvR*
320 (37), *fimA* (38), and *viaB* (39). However, they have shortcomings for the identification
321 of *Salmonella* species, and false-positives limit the diagnostic process. At present,
322 quorum sensing-related such as *luxS* and *gyrB* are widely used, along with virulence
323 genes *invH*, *sopE*, *hilA*, and *invA* in the SP11 pathogenicity island, *sugR*, *rhuM*, and
324 *iacP* in the SPI-3 pathogenicity island, and *spvB* and *spvC* in virulence plasmids. Of
325 these, *invA*, which encodes an epithelial cell surface protein, is present in all
326 *Salmonella* species, and is the most widely reported target gene in the *Salmonella*
327 genus. Primers and probes for the diagnosis of *Salmonella* spp. based on this
328 conserved gene are highly specific, hence *invA* was selected as the target gene for
329 real-time qPCR detection of *Salmonella* species in the present study.

330

331 The 16S rDNA gene has been used for classification and identification of *Serratia*
332 species in previous studies, as has the quorum sensing gene *luxS* (17), which was
333 employed in real-time qPCR (21). In addition, several studies used carbapenem
334 antibiotic resistance genes for identification of *Serratia* species (40). The *gyrB* gene,
335 encoding the B subunit of DNA gyrase (GyrB) that forms topoisomerase II, an
336 essential protein in replication, transcription, DNA synthesis, and maintenance of the
337 DNA supercoiled structure, is more reliable than the 16S rDNA gene for the
338 identification of bacterial species (41). The protein-coding *gyrB* gene contains more
339 genetic information than the non-protein-coding 16S rDNA, resulting in a greater
340 capacity to distinguish bacterial species (42). Thus, the *gyrB* gene was selected for
341 discriminating *S. fonticola* and *Salmonella* spp. using real-time qPCR.

342

343 Real-time qPCR requires stricter primers, amplification products, and reaction
344 conditions than conventional PCR. Primers used for real-time qPCR are typically
345 18–30 bp in length. Shorter primers (<15 nucleotides) can be combined
346 efficiently, but often to the detriment of specificity. By contrast, longer primers
347 display enhanced specificity but may also hybridize with the wrong pairing sequence,
348 reducing specificity and decreasing the efficiency of hybridization, resulting in
349 diminished PCR amplification. Ideal results are generally obtained when amplifying

350 products of less than 300 bp. Therefore, the PCR products selected for differentiating
351 *Salmonella* spp. and *S. fonticola* were 199 bp and 94 bp, respectively. Different
352 annealing temperatures were tested to determine the optimum annealing temperature
353 of the two pairs of primers, in order to reduce the influence of annealing temperature
354 on the duplex real-time qPCR experiment. An annealing temperature of 64°C was
355 found to be optimal.
356
357 The duplex real-time qPCR approach established for the rapid detection of *Salmonella*
358 spp. and *S. fonticola* in imported feedstuffs was characterized by a high correlation
359 coefficient between the Ct value and the logarithm of the initial copy number ($R^2 =$
360 0.999). Additionally, parameters including slope, y-axis intercept, and amplification
361 efficiency between duplex real-time PCR and single real-time PCR were compared.
362 As shown in Table 5, the slope of the standard curve and its intercept on the y-axis are
363 approximately equal, and the amplification efficiency is ~100%. Thus, simultaneous
364 detection of the two pathogens was achieved in a duplex real-time qPCR
365 amplification assay, and the detection limit of this method is suitable for daily
366 inspection and quarantine work. In summary, the rapid diagnostic method established
367 in this study has many advantages, including a low detection limit and high
368 repeatability. It is also rapid and convenient to deploy, since the results can be
369 obtained within several hours after pre-enriching, representing a significant
370 improvement in efficiency for detecting *Salmonella* spp. in imported animal-derived
371 feedstuffs during quarantine work. This method is of great theoretical and practical
372 value for ensuring the safety of imported feedstuffs, and could help to prevent
373 adventive pathogens by facilitating effective quarantining and disposal measures.
374

375 **Conflict of interests**
376 The authors declare no competing interests.
377

378 **Authors and Contributors**
379 Conceived and designed the experiments: Jh R, Wj W, and Dj Y. Performed the
380 experiments: Jh R. Generated and analyzed the data: Jh R, Wj W, Qy B, TZ, and Zd Z.
381 Collected the clinical isolates: Ty Z, Sy Y, and Sj L. Wrote the paper: Jh R. Revised
382 the paper: Dj Y and Bx Z. All authors read and approved the final manuscript.
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References

1. Gavini F, Ferragut C, Izard D, Trinel PA, Leclerc H, Lefebvre B, Mossel DAA. 1979. Serratia fonticola, a New Species from Water. Int J Syst Bacteriol 29:92-101.

2. Tasic S, Obradovic D, Tasic I. 2013. Characterization of Serratia fonticola, an opportunistic pathogen isolated from drinking water. Arch Biol Sci 65:899-904.

3. Rd FJ, Davis BR, Hickmanbrenner FW, Mcwhorter A, Huntleycarter GP, Asbury MA, Riddle C, Wathengrady HG, Elias C, Fanning GR. 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. J Clin Microbiol 21:46.

4. Bollet C, Gainnier M, Sainty JM, Orhesser P, Micco PD. 1991. Serratia fonticola isolated from a leg abscess. J Clin Microbiol 29:834-835.

5. Gorret J, Chevalier J, Gaschet A, Fraisse B, Violas P, Chapuis M, Jolivet-Gougeon A. 2009. Childhood delayed septic arthritis of the knee caused by Serratia fonticola. Knee 16:512.

6. Garcia ME, Lanzarot P, Costas E, Lopez RV, Marín M, Blanco JL. 2008. Isolation of Serratia fonticola from skin lesions in a Nile Crocodile (Crocodylus niloticus) with an associated septicaemia. Vet J 176:254.

7. Stock I, Burak S, Sherwood KJ, Gruger T, Wiedemann B. 2003. Natural antimicrobial susceptibilities of strains of 'unusual' Serratia species: S. ficaria, S. fonticola, S. odorifera, S. plymuthica and S. rubidaea. J Antimicrob Chemother 51:865.

8. Aljorayid A, Viau R, Castellino L, Jump RLP. 2016. Serratia fonticola, pathogen or bystander? A case series and review of the literature. Idcases 5:6-8.

9. Soler C, Samson T, Hernandez E, Hervé V, Revel TD. 2000. Serratia fonticola, germe opportuniste: à propos d'une observation chez un immunodéprimé. Med Mal Infect 30:599-600.

10. Kunimoto D, Rennie R, Citron DM, Goldstein EJ. 2004. Bacteriology of a bear bite wound to a human: case report. J Clin Microbiol 42:3374.

11. Chen Y, Wu D, Sun M, Deng M, Cui S, Liang C, Geng J, Sun T, Long L, Xiao X. 2016. Serum Bactericidal Assay: New Role in Salmonella Detection. J AOAC Int 99:124.

12. Jain S, Chattopadhyay S, Jackeray R, Abid Z, Singh H. 2016. Detection of Salmonella typhi utilizing bioconjugated fluorescent polymeric nanoparticles. J Nanopart Res 18:1-11.

13. Kao CC, Liu MF, Lin CF, Huang YC, Liu PY, Chang CW, Shi ZY. 2010. Antimicrobial susceptibility and multiplex PCR screening of AmpC genes

438   from isolates of Enterobacter cloacae, Citrobacter freundii, and Serratia
439   marcescens. Journal of Microbiology, Immunol Infect 43:180-187.

440   14.   Machado, Goncalves S, Bazzolli S, Mara D, Vanetti D, Cristina M. 2013.
441   Development of a PCR method for detecting proteolytic
442   psychrotrophic;bacteria in raw milk. Int Dairy J 29:8-14.

443   15.   Rahn K, De Grandis SA, Clarke RC, Mcewen SA, Galán JE, Ginocchio C, Rd
444   CR, Gyles CL. 1992. Amplification of an invA gene sequence of Salmonella
445   typhimurium by polymerase chain reaction as a specific method of detection
446   of Salmonella. Mol Cell Probes 6:271.

447   16.   Van dBM, Peters RP, Catsburg A, Rubenjan A, Broeke FJ, Fa VDD, van
448   Weissenbruch MM, van Furth AM, Kõressaar T, Remm M. 2014.
449   Development of a multiplex real-time PCR assay for the rapid diagnosis of
450   neonatal late onset sepsis. J Microbiol Methods 106:8.

451   17.   Zhu H, Sun SJ, Dang HY. 2008. PCR detection of Serratia spp. using primers
452   targeting pfs and luxS genes involved in AI-2-dependent quorum sensing. Curr
453   Microbiol 57:326-330.

454   18.   Hurst MRH, Young SD, O'Callaghan M. 2008. Development of a
455   species-specific probe for detection of Serratia entomophila in soil. N
456   Z Plant Prot 61:222-228.

457   19.   Cornegliani L, Corona A, Vercelli A, Roccabianca P. 2015. Identification by
458   real-time PCR with SYBR Green of Leishmania spp. and Serratia marcescens
459   in canine 'sterile' cutaneous nodular lesions. Vet Dermatol 26:186–e138.

460   20.   Iwaya A, Nakagawa S, Iwakura N, Taneike I, Kurihara M, Kuwano T,
461   Gondaira F, Endo M, Hatakeyama K, Yamamoto T. 2005. Rapid and
462   quantitative detection of blood Serratia marcescens by a real-time PCR assay:
463   its clinical application and evaluation in a mouse infection model. Fems
464   Microbiol Lett 248:163.

465   21.   Joyner J, Wanless D, Sinigalliano CD, Lipp EK. 2014. Use of Quantitative
466   Real-Time PCR for Direct Detection of Serratia marcescens in Marine and
467   Other Aquatic Environments. Appl Environ Microbiol 80:1679.

468   22.   Nam HM, Srinivasan V, Gillespie BE, Murinda SE, Oliver SP. 2005.
469   Application of SYBR green real-time PCR assay for specific detection of
470   Salmonella spp. in dairy farm environmental samples. Int J Food Microbiol
471   102:161-171.

472   23.   Perelle S, Dilasser F, Malorny B, Grout J, Hoorfar J, Fach P. 2004.
473   Comparison of PCR-ELISA and LightCycler real-time PCR assays for
474   detecting Salmonella spp. in milk and meat samples. Mol Cell Probes 18:409.

475   24.   Tomás D, Rodrigo A, Hernández M, Ferrús MA. 2009. Validation of
476   Real-Time PCR and Enzyme-Linked Fluorescent Assay-Based Methods for
477   Detection of Salmonella spp. in Chicken Feces Samples. Food Anal Methods
478   2:180-189.

479   25.   Elizaquível P, Aznar R. 2008. A multiplex RTi-PCR reaction for simultaneous
480   detection of Escherichia coli O157:H7, Salmonella spp. and Staphylococcus
481   aureus on fresh, minimally processed vegetables. Food Microbiol 25:705.

26. Omiccioli E, Amagliani G, Brandi G, Magnani M. 2009. A new platform for Real-Time PCR detection of Salmonella spp., Listeria monocytogenes and Escherichia coli O157 in milk. Food Microbiol 26:615-622.

27. Suo B, He Y, Tu SI, Shi X. 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of Salmonella spp., Escherichia coli O157, and Listeria monocytogenes in meat products. Foodborne Pathog Dis 7:619.

28. Wang L, Li Y, Mustaphai A. 2007. Rapid and simultaneous quantitation of Escherichia coli 0157:H7, Salmonella, and Shigella in ground beef by multiplex real-time PCR and immunomagnetic separation. J Food Prot 70:1366.

29. Aiyar A. 2000. The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. Methods Mol Biol 132:221.

30. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389.

31. Jing HR, Wu JW, Zhang TY, Quan YB, Teng Z, Zhi DZ, Li YW, Yi FH, Yu DJ. 2017. Rapid Detection of Serratia fonticola by TaqMan Quantitative Real-Time PCR Using Primers Targeting the gyrB Gene. Curr Microbiol 74: 1343–1348. DOI 10.1007/s00284-017-1323-x

32. Whelan JA, Russell NB, Whelan MA. 2003. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods 278:261-269.

33. Crăciunaş C, Keul AL, Flonta M, Cristea M. 2012. DNA-based diagnostic tests for Salmonella strains targeting hilA , agfA , spvC and sef genes. J Environ Manage 95:S15.

34. Mann E, Hein I, Mester P, Stessl B, Rossmanith P, Wagner M, Dzieciol M. 2013. A Robust and Poisson Validated Quantitative 5′ Nuclease TaqMan® Real-Time PCR Assay Targeting fimA for the Rapid Detection of Salmonella spp. in Food. Food Anal Methods 6:991-995.

35. Shanmugasundaram M, Radhika M, Murali HS, Batra HV. 2009. Detection of Salmonella enterica serovar Typhimurium by selective amplification of fliC, fljB, iroB, invA, rfbJ, STM2755, STM4497 genes by polymerase chain reaction in a monoplex and multiplex format. World J Microbiol Biotechnol 25:1385-1394.

36. Ngan GJ, Ng LM, Lin RT, Teo JW. 2010. Development of a novel multiplex PCR for the detection and differentiation of Salmonella enterica serovars Typhi and Paratyphi A. Res Microbiol 161:243-248.

37. Mahon J, Lax AJ. 1993. A Quantitative Polymerase Chain Reaction Method for the Detection in Avian Faeces of Salmonellas Carrying the spvR Gene. Epidemiol Infect 111:455.

38. Cano RJ, Rasmussen SR, Sánchez FG, Palomares JC. 1993. Fluorescent detection-polymerase chain reaction (FD-PCR) assay on microwell plates as a screening test for salmonellas in foods. J Appl Bacteriol 75:247-253.

39. Hashimoto Y, Itho Y, Fujinaga Y, Khan AQ, Sultana F, Miyake M, Hirose K,

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526   Yamamoto H, Ezaki T. 1995. Development of nested PCR based on the ViaB
527   sequence to detect Salmonella typhi. J Clin Microbiol 33:302–318.

528 40.   Yamamoto S, Harayama S. 1996. Phylogenetic analysis of Acinetobacter
529   strains based on the nucleotide sequences of gyrB genes and on the amino acid
530   sequences of their products. Int J Syst Bacteriol 46:506.

531 41.   Gellert M, Mizuuchi K, O'Dea MH, Nash HA. 1976. DNA gyrase: an enzyme
532   that introduces superhelical turns into DNA. Proc Natl Acad Sci U S
533   A 73:3872.

534 42.   Kasai H, Watanabe K, Gasteiger E, Bairoch A, Isono K, Yamamoto S,
535   Harayama S. 1998. Construction of the gyrB Database for the Identification
536   and Classification of Bacteria. Genome Inform Ser Workshop Genome Inform
537   9:13.

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Supplementary figure legends

FIGURE 1 Specificity testing of the real-time qPCR assay using 29 *Salmonella* spp. strains.

FIGURE 2 Specificity testing of the real-time qPCR assay using four *Serratia fonticola* strains.

FIGURE 3 Standard curves for *Salmonella* spp. real-time qPCR assay. The curves represent a range from 10^{10} to 10^2 copies per reaction.

FIGURE 4 Standard curves for *S. fonticola* real-time qPCR assay. The curves represent a range from 10^{10} to 10^2 copies per reaction.

FIGURE 5 Sensitivity of the real-time qPCR assay for the detection of *Salmonella* species.
Amplification plots from left to right represent a range of *invA* gene-containing plasmids from 1.97×10^{10} to 1.97×10^2 copies/µL, respectively. Amplification plot 10 is a negative control.

FIGURE 6 Sensitivity of the real-time qPCR assay for the detection of *S. fonticola*. Amplification plots from left to right represent a range of *gyrB* gene-containing plasmid from 1.45×10^{10} to 1.45×10^2 copies/µL, respectively. Amplification plot 10 is a negative control.

FIGURE 7 Amplification plot for the duplex real-time qPCR assay using serially diluted plasmids. Amplification plots 1–5 represent *gyrB* gene-containing plasmid ranging from 1.97×10^{10} to 1.97×10^6 copies/µL, respectively. Amplification plots 6–10 represent *invA* gene-containing plasmid ranging from 1.45×10^{10} to 1.45×10^6 copies/µL, respectively.

FIGURE 8 Standard curves for the duplex RT-qPCR assay. Y_1 and Y_2 are standard curves using *invA* gene- and *gyrB* gene-containing plasmids, respectively.

13

571

572 **TABLE 1 *Salmonella* spp. strains used in this study**

Bacterial species	Strain No.	No. of strains	Source of strains
Salmonella enteritidis	ATCC13076	1	ATCC (Manassas, USA)
Salmonella typhimurium	GDM1.237	1	GDMCC (Guangdong, China)
Salmonella montevideo	/	2	Imported fishmeal
Salmonella weltevreden	/	1	Imported fishmeal
Salmonella enteritidis	/	3	Imported fishmeal
Salmonella birkenhead	/	1	Imported fishmeal
salmonella anatis	/	1	Imported fishmeal
Salmonella senftenberg	/	1	Imported fishmeal
Salmonella lomita	/	1	Imported fishmeal
Salmonella saintpaul	/	1	Imported fishmeal
Salmonella O:2	/	3	Imported fishmeal
Salmonella O:4	/	1	Imported fishmeal
Salmonella O:7	/	2	Imported fishmeal
Salmonella O:8	/	2	Imported fishmeal
Salmonella O:9	/	3	Imported fishmeal
Salmonella O:3,10	/	5	Imported fishmeal

573

574 **TABLE 2 *Serratia* genus strains used in this study**

Bacterial species	Strain No.	No. of strains	Source of strains
Serratia fonticola	GDM1.995	1	GDMCC (Guangdong, China)
Serratia marcescens	MCCC1A06806	1	MCCC (Xiamen, China)
Serratia proteamaculans	MCCC1K00532	1	MCCC (Xiamen, China)
Serratia odorifera	GDM1.864	1	GDMCC (Guangdong, China)
Serratia ficaria	GDM1.994	1	GDMCC (Guangdong, China)
Serratia plymuthica	GDM1.996	1	GDMCC (Guangdong, China)
Serratia rubidaea	GDM1.1008	1	GDMCC (Guangdong, China)
Serratia liquefaciens	CICC21538	1	CICC (Beijing, China)
Serratia grimesii	ACCC01695	1	ACCC (Beijing, China)
Serratia fonticola	/	3	Imported fishmeal

575

576 **TABLE 3 Other strains used in this study**

Bacterial species	Strain No.	No. of strains	Source of strains
Eschericha coli	ATCC25922	1	ATCC (Manassas, USA)
Citrobacter freundii	ATCC8090	1	ATCC (Manassas, USA)
Shigella sonnei	ATCC25931	1	ATCC (Manassas, USA)
Klebsiella oxytoca	ATCC700324	1	ATCC (Manassas, USA)
Enterrococcus faecalis	ATCC29212	1	ATCC (Manassas, USA)
Enterobacter aerogenes	ATCC13048	1	ATCC (Manassas, USA)
Klebsiella pneumoniae	20161226-16	1	Imported fishmeal

577 MCCC, Marine Culture Collection of China; GDMCC, Guangdong Microbial Culture Center; CICC,

578 China Center of Industrial Culture Collection; ACCC, Agricultural Culture Collection of China; ATCC,

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579   American Type Culture Collection; GDMCC, Guangdong Microbial Culture Center.

580

581   **TABLE 4 Real-time qPCR primer pairs and probes used in this study**

Primers and probes	Length of product
invA -F: 5'-ATGGAAGCGCTCGCATTGTG-3'	
invA -R: 5'-GGCTGAGGAAGGTACTGCCA-3'	199 bp
invA -P: 5'-JOE-TGCTCGTAATCCGCCGCCATTGGCG-BHQ1-3'	
gyrB -F: 5'-TCGGTGAAACCGATCAGAC-3'	
gyrB -R: 5'-GCCAGGATGTCGTACTCAAA-3'	94 bp
gyrB -P: 5'-6 FAM-CTGCGCTTCTGGCCGAGCTT-BHQ1-3'	

582

583   **TABLE 5 Comparison of parameters between duplex and single real-time PCR assays**

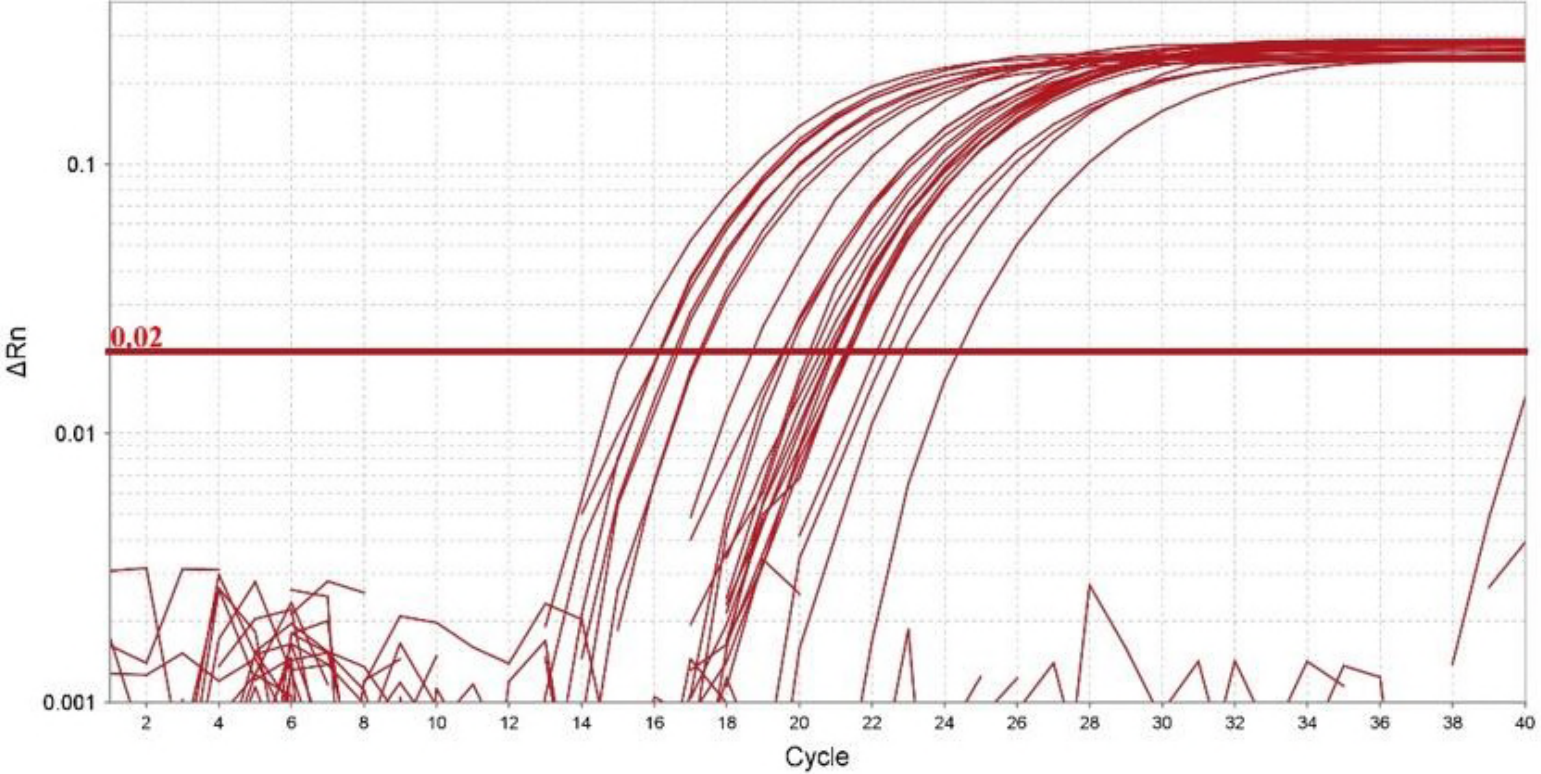| Plasmid	Slope		y-axis intercept		R^2		Eficiency（%）	
	Single	Duplex	Single	Duplex	Single	Duplex	Single	Duplex
pUCm-*invA*	-3.267	-3.362	48.116	48.775	0.998	0.999	102.344	98.346
pUCm-*gyrB*	-3.242	-3.409	42.919	44.396	0.999	0.999	103.429	96.49

584

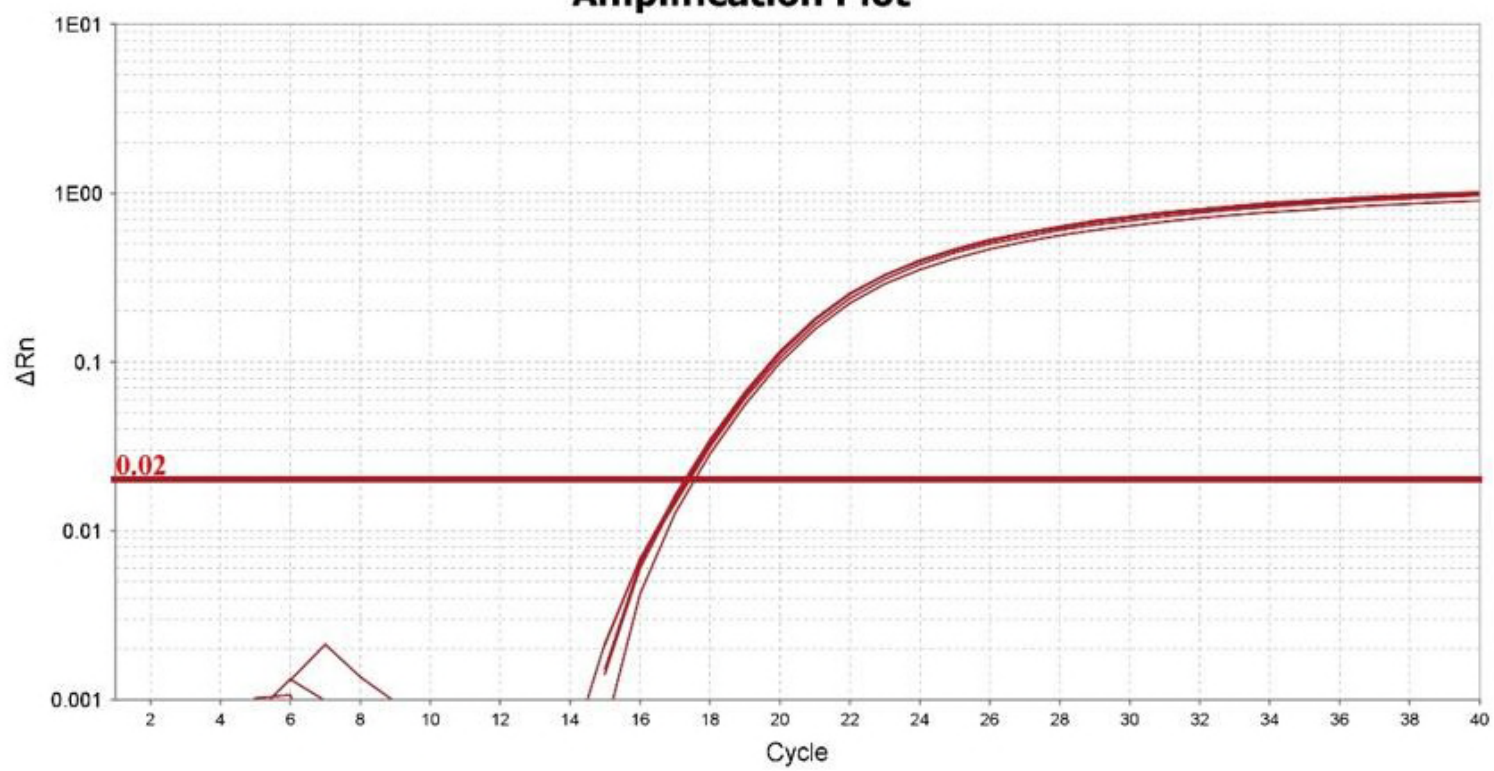585   **TABLE 6 Reproducibility and stability testing of the duplex real-time PCR**

Ten-fold dilution (copies/μL）	C_t value			$Ct_{MN \pm SD}$	CV (%)
	1	2	3		
1.97E+08	19.989	20.086	20.195	20.090 ± 0.103	0.51
1.97E+07	23.287	23.331	23.302	23.307 ± 0.022	0.09
1.97E+06	26.924	26.861	26.879	26.879 ± 0.040	0.15
1.97E+05	29.892	29.934	29.910	29.910 ± 0.022	0.07
1.97E+04	33.251	33.525	33.356	33.377 ± 0.139	0.42
1.45E+08	15.997	16.005	15.856	15.952 ± 0.084	0.53
1.45E+07	19.581	19.330	19.343	19.418 ± 0.141	0.73
1.45E+06	22.909	22.724	22.898	22.844 ± 0.104	0.46
1.45E+05	25.522	25.526	25.628	25.559 ± 0.060	0.23
1.45E+04	28.918	29.209	29.146	29.091 ± 0.153	0.53

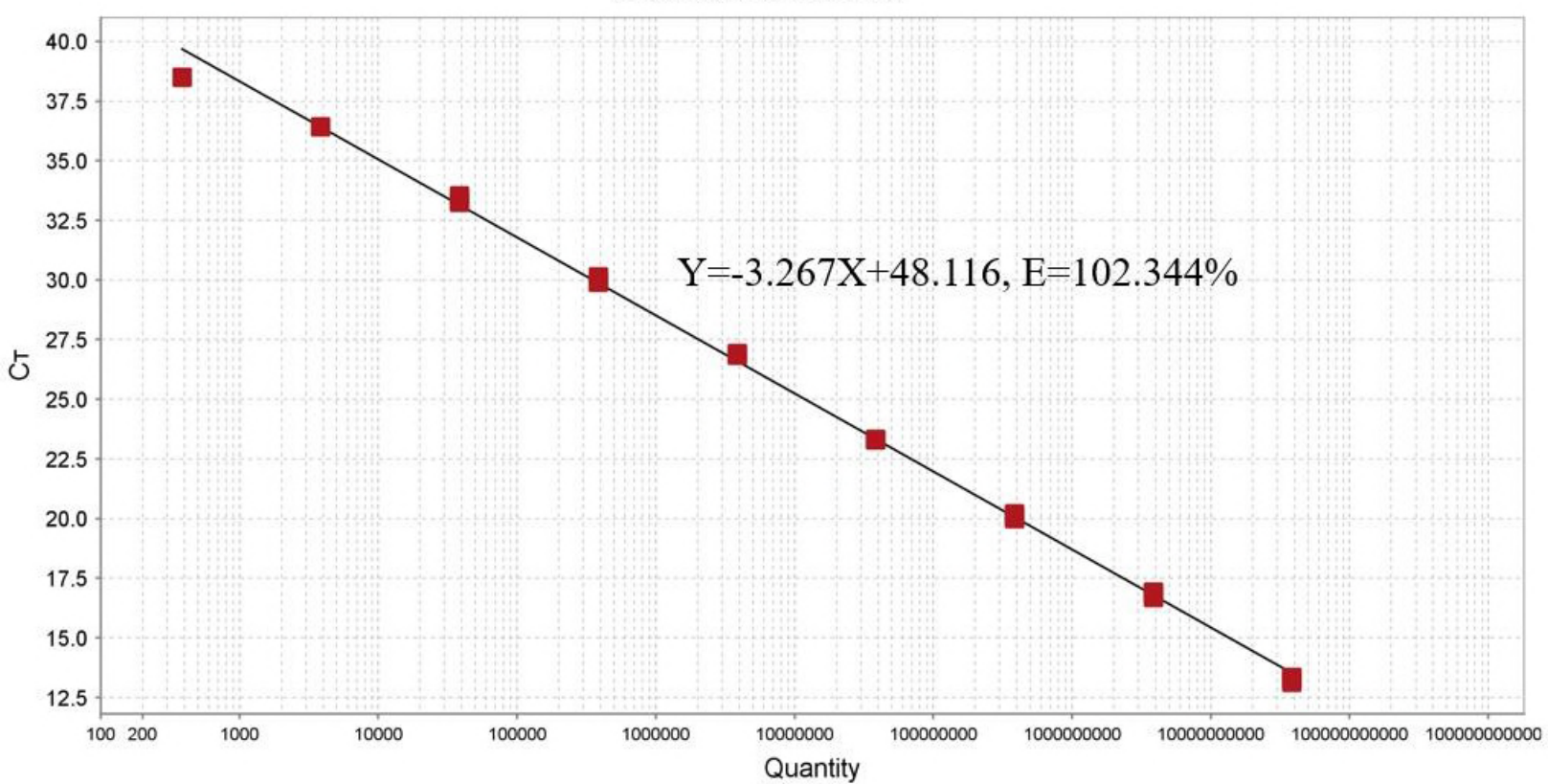586   SD, Standard Deviation; MN, Mean; CV (Coefficient of Variance) = (SD / MN) × 100%
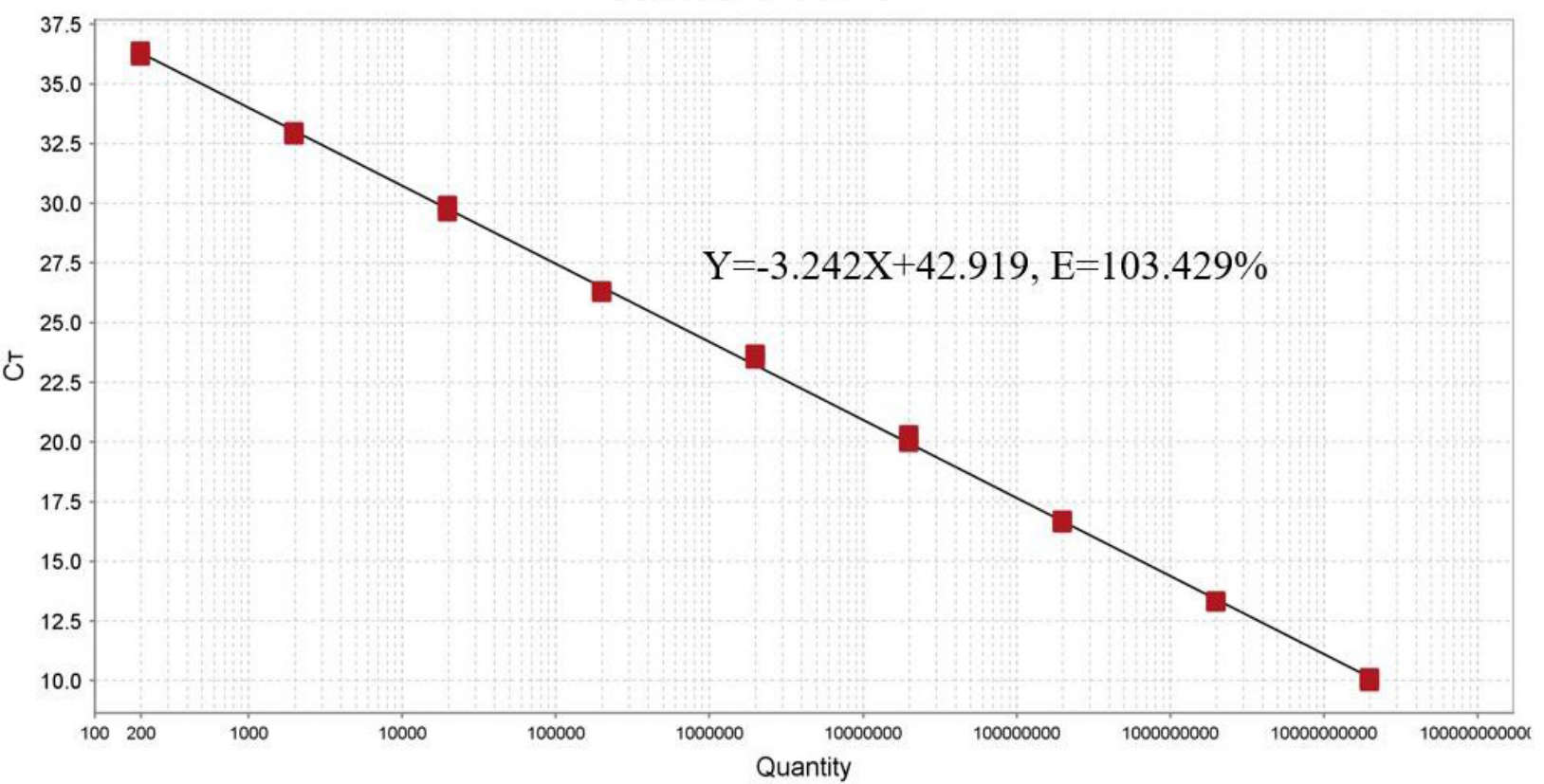
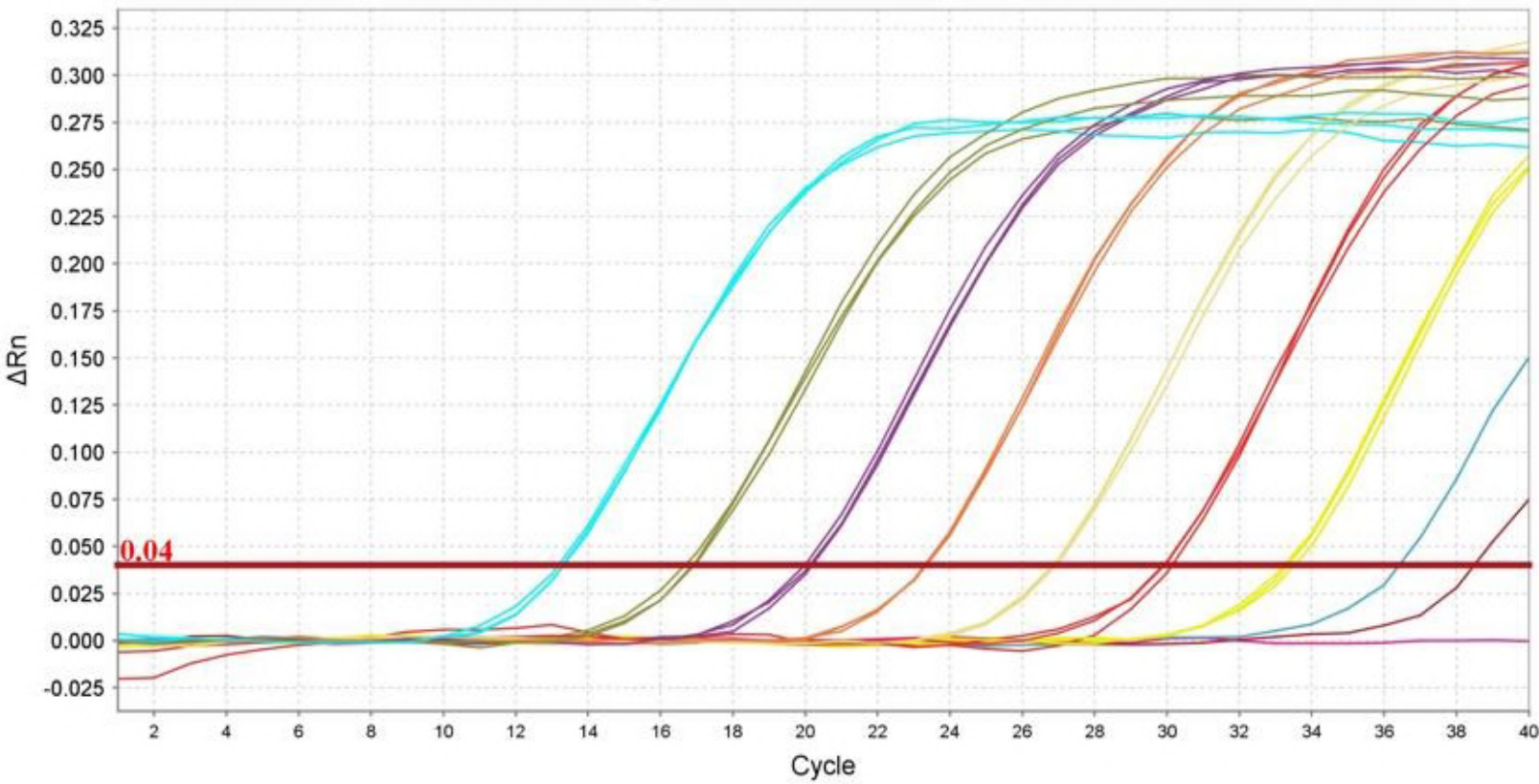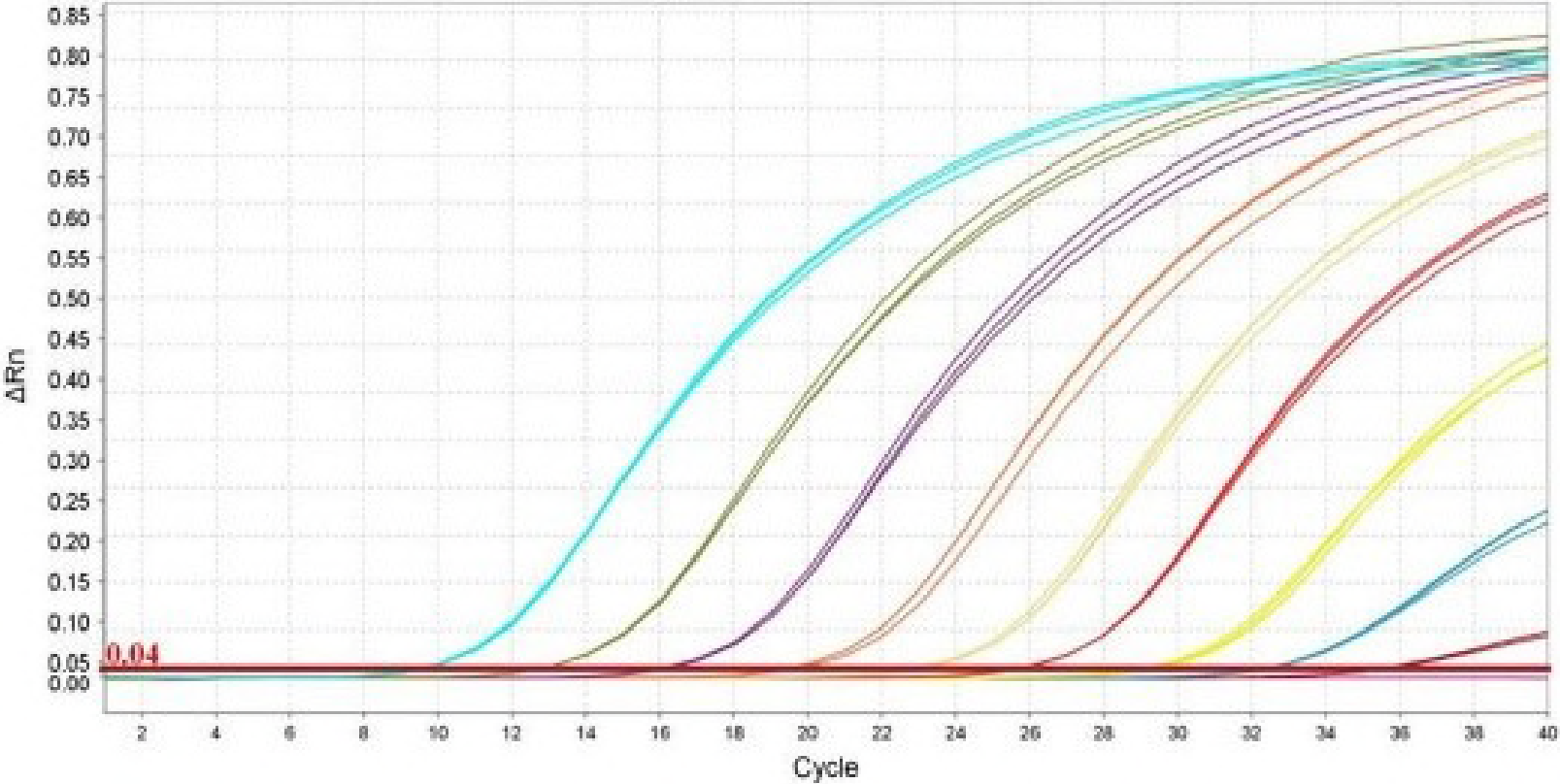Amplification Plot

Amplification Plot

Standard Curve

Y=-3.267X+48.116, E=102.344%

Standard Curve

Y=-3.242X+42.919, E=103.429%

Amplification Plot

Amplification Plot

Standard Curve