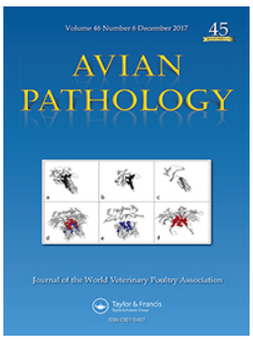

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ORIGINAL ARTICLE

Development of a multiplex qPCR in real time for quantification and differential diagnosis of *Salmonella* Gallinarum and *Salmonella* Pullorum

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ABSTRACT

Currently there are 2659 *Salmonella* serovars. The host-specific biovars *Salmonella* Pullorum and *Salmonella* Gallinarum cause systemic infections in food-producing and wild birds. Fast diagnosis is crucial to control the dissemination in avian environments. The present work describes the development of a multiplex qPCR in real time using a low-cost DNA dye (SYBr Green) to identify and quantify these biovars. Primers were chosen based on genomic regions of difference (RoD) and optimized to control dimers. Primers pSGP detect both host-specific biovars but not other serovars and pSG and pSP differentiate biovars. Three amplicons showed different melting temperatures (*Tm*), allowing differentiation. The pSGP amplicon (97 bp) showed *Tm* of 78°C for both biovars. The pSG amplicon (273 bp) showed a *Tm* of 86.2°C for *S.* Gallinarum and pSP amplicon (260 bp) dissociated at 84.8°C for *S.* Pullorum identification. The multiplex qPCR in real time showed high sensitivity and was capable of quantifying 10^8–10^1 CFU of these biovars.

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KEYWORDS
Fowl typhoid; pullorum disease; SYBr green; melting curve; ROD; identification; biovar

Introduction

Salmonella enterica belongs to the family Enterobacteriaceae and, currently, 2659 different serovars have been identified (Issenhuth-Jeanjean *et al.*, 2014). All serovars are pathogenic. However, diseases in birds are classified in three different categories. The flagellated serovars, which are the vast majority (e.g. *S.* Enteritidis and *S.* Typhimurium), cause paratyphoid infections in different hosts including humans, mammals, reptiles and birds, with pronounced gastrointestinal colonization (Barletta *et al.*, 2013). Poultry are susceptible to paratyphoid infections and are frequently associated as source of infection in foodborne disease in humans through the consumption of contaminated meat or eggs (Omiccioli *et al.*, 2009).

The other two categories of diseases in birds are fowl typhoid caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S.* Gallinarum) and pullorum disease caused by *Salmonella enterica* serovar Gallinarum biovar Pullorum (*S.* Pullorum). Both biovars are closely related genetically, non-motile and are host-specific pathogens of birds (Löfström *et al.*, 2010; Feng *et al.*, 2013). Infections or outbreaks are of compulsory notification to the World Organisation for Animal Health (OIE), due to the mortality of animals, losses caused and risk of dissemination to other countries.

S. Pullorum affects newly hatched young chicks, causing septicaemic colonization, with high mortality in affected flocks. Infection initiates mainly from vertical transmission originating from subclinically infected adult breeders. However, horizontal transmission contributes to fast dissemination within the flock. *S.* Gallinarum is horizontally transmitted and evidence points to a vertical dissemination route (Celis-Estupinan *et al.*, 2017). Moreover, the infection causes high morbidity and up to 80% of mortality in young or adult chickens (Shivaprasad, 2000). Different factors, such as the interaction with the intestinal epithelium, high invasiveness, intracellular survival in infected macrophages and evasion of the immune response, are involved in the pathogenesis of these bacteria (Soria *et al.*, 2013).

The gold standard method used for the diagnosis of *S.* Gallinarum and *S.* Pullorum during outbreaks is microbiological culture, for isolation, biochemical identification and serotyping of the isolate (Santana *et al.*, 2011; Ma *et al.*, 2014). In addition to direct methods used to isolate these bacteria, indirect methods are important for epidemiological studies and monitoring of the sanitary conditions in flocks. Serological tests such as ELISA and rapid sero-agglutination test can detect antibodies against *Salmonella* spp. and PCR is capable of detecting and identifying the DNA of the pathogen (Andrade *et al.*, 2010).

The bacterial culture of *Salmonella* spp. has a few disadvantages compared to molecular biology techniques. Among these, the direct identification of the bacteria is difficult due to the growth of other microorganisms present in the sample, especially from

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microbiota. The complete identification depends on biochemical and serological analysis of each isolate, increasing the time and cost to obtain results with this method (Chen *et al.*, 2010). The diagnosis by ELISA technique produces results within two days. However, this technique relies on the detection of antibodies and may require confirmation by conventional microbiology, which prolongs obtaining a diagnosis (Dickel *et al.*, 2005).

The PCR technique has been successfully established for fast and reliable diagnosis of different pathogens, including fastidious bacteria. The results may be obtained within two days or less. However, positive results do not require the confirmation by conventional microbiology. The result analysis follows an established pattern of interpretation (Lin *et al.*, 2011). Many epidemiological surveys were successfully conducted during the last decades using PCR, because this method can be designed for high specificity and sensitivity (Chen *et al.*, 2010).

Real-time PCR has been more recently developed for diagnosis of pathogens, with the features of higher sensitivity than conventional PCR and faster protocols to obtain the results. Thus, in the present study, we describe the development of a multiplex real-time PCR to specifically detect and differentiate closely related biovars *S.* Gallinarum and *S.* Pullorum and simultaneously quantify the bacterial numbers in samples.

Material and methods

Bacterial strains for validation of the real-time PCR assay

Twenty-eight isolates from biovar *S.* Gallinarum and 18 isolates from biovar *S.* Pullorum were used for DNA extraction, development and validation of the multiplex quantitative PCR in real time, including the sequenced strains *S.* Gallinarum SG9 (accession number: CM001153.1), *S.* Gallinarum 287/91 (a.n.: AM933173.1), *S.* Pullorum FCAV198 (a.n.: AZRG00000000). Additionally, DNA from 59 different *Salmonella* isolates (including non-typhoidal and typhoidal serovars) and six other Enterobacteriaceae (Supplemental Table 1) were used as negative controls for validation of the primers' specificity. Isolates were obtained from two National Reference Laboratories (Supplemental Table 1) or from the Laboratory of Avian Pathology at the School of Agricultural and Veterinary Sciences (FCAV-Unesp/Jaboticabal/SP) and stored at −80°C.

Bacterial DNA extraction

Each isolate was cultured in Luria-Bertani broth (LB) at 37°C, shaking at 100 rpm for a period of 18 hours, including the bacteria used as negative controls. Before DNA extraction, all *S.* Gallinarum and *S.* Pullorum cultures were quantified by plating serial dilutions in Brilliant Green agar (Oxoid CM0263; Oxoid Ltd, Basingstoke, UK) of the culture with subsequent incubation at 37°C for 24 h. After incubation, the bacterial numbers of each culture were evaluated and the colony forming units per ml (CFU/ml) were transformed to Log_{10}. DNA extraction of all bacterial cultures was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity and concentration of bacterial DNA was analysed by spectrophotometry (Nanodrop 1000 Thermo Fisher Scientific, Waltham, MA, USA).

Primer design for differential diagnosis between S. Gallinarum and S. Pullorum

Three pairs of primers were used and the sequences and details are in Table 1. The primer pSGP was designed to detect the *S. enterica* serovar Gallinarum, including both biovars, without detection of other typhoidal or non-typhoidal *Salmonella* sp. serovars. The primers pSG and pSP were designed to differentiate *S.* Gallinarum biovar Gallinarum (pSG) and *S.* Gallinarum biovar Pullorum (pSP) based on the analysis of regions of difference (RoDs) from each biovar (Batista *et al.*, 2015). Primers were designed using the Primer 3 software (Free Software Foundation, Boston, MA, USA), based on the complete genome sequence of *S.* Gallinarum and *S.* Pullorum available at the NCBI database (http://www.ncbi.nlm.nih.gov/genome/) and validated *in silico* using the primer BLAST tool, to check for non-specific annealing with other sequenced microorganisms.

Cloning of the target DNA amplicon

The target genome regions used for the specific amplification and diagnosis of each biovar were cloned into plasmids for use as positive controls and to obtain the quantitative standard curve in the real-time PCR. Briefly, the amplicons obtained after conventional PCR using the primers pSG for *S.* Gallinarum and pSP for *S.* Pullorum were digested with BamHI and subsequently cloned into plasmids. After cloning, the accurate copy number of the target amplicons was calculated and the DNA of each clone was diluted, corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU of *S.* Gallinarum or *S.* Pullorum, to obtain the quantitative standard curve.

Conditions of the qPCR in real time

The multiplex real-time PCR was performed using the following optimized reaction mix of 13 µl Luminoct® SYBr® Green qPCR Readymix (Sigma-Aldrich, St. Louis, MI, USA), containing 1.25 U of Taq DNA

Table 1. Specifications of the primers used in the study.

Primer	Sequence	Product size (bp)	Concentration (μM)	Amplicon *Tm*
pSGP F	TGGCCTTGACGTTATTCGTT	97	0.1	78.0°C
pSGP R	AGCATTTTGGTCACCTGCAA			
pSG F	ATGGCGAGTCCGCCCAGAGT	273	0.05	86.2°C
pSG R	GGCGGTATGCTGGTTGCCGT			
pSP F	CCGCCTGCGCGATGGCTTTA	260	0.05	84.8°C
pSP F	TCTGGTTGACGGCGTGGGGA			

Note: Primers pSG were designed based on ROD43 and primers pSP were designed based on ROD24 (Batista, 2013).

polymerase and adding 0.1 μM of the primer pSGP, 0.05 μM of primers pSG and pSP, 2.5 μl of serially diluted DNA or eluted DNA after extraction from tested samples, and ultrapure water to make the final volume 25 μl. The first dilution (10^{-1}) corresponded to 100 ng of DNA and the following dilutions were made in $\log_{10}$ up to 10^{-8} from the initial DNA sample. The qPCR in real time was performed in the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA), with cycling protocol starting with one denaturation cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 15 s and 63°C for 30 s. After amplification, the melting curve was performed to obtain the specific melting temperature (*Tm*) of each amplicon for the differential diagnosis between *S.* Gallinarum and *S.* Pullorum.

Standard curve for bacterial quantification

Serially diluted plasmid DNA containing the cloned DNA from biovars *S.* Gallinarum and *S.* Pullorum was used to prepare the quantitative standard curve. The reactions followed the optimized PCR conditions for the multiplex reactions. The Software CFX Manager™ 3.1 (Bio-Rad) was used to calculate the regression coefficients, standard deviation and standard curves efficiency ratio. The linear standard curve was used for quantification of number of genes copies that corresponded to the number of CFU of each biovar. The reactions were acceptable for analysis when R^2 value was higher than 0.99.

Results and discussion

The genus *Salmonella* spp. has often been reported in studies using different microbiological and molecular techniques for the identification and differentiation in samples from company and food-producing animals (Dickel *et al.*, 2005; Malorny *et al.*, 2007; O'Regan *et al.*, 2008; Mcguinness *et al.*, 2009; Omiccioli *et al.*, 2009; Andrade *et al.*, 2010; Löfström *et al.*, 2010; Park *et al.*, 2011; Barletta *et al.*, 2013; Dobhal *et al.*, 2014; Li *et al.*, 2014; Ma *et al.*, 2014). The fast diagnosis and identification of these bacteria is crucial for the control of bacterial dissemination, reduction of losses in animal production and risks to human health caused by foodborne infections (Chen *et al.*, 2010; Cheraghchi *et al.*, 2014).

Currently there are 2659 pathogenic serovars identified and most cause gastrointestinal infections in a large number of hosts (Issenhuth-Jeanjean *et al.*, 2014). However, a restricted number of serovars are host-specific. Considering the high pathogenicity of the host-specific biovars *S.* Gallinarum and *S.* Pullorum to birds, the differentiation from others is important to take the correct measures in commercial poultry farms. In Brazil, the presence of either of these two biovars in breeder flocks of chickens condemns the entire flock to elimination, sanitation and replacement of the birds. Thus, the diagnosis has to be fast and accurate, to avoid false positives, as the presence of other non-typhoidal serovars allow treatments and milder biosafety measures in these flocks.

Different PCR methods have been described for the differential diagnosis of a few *Salmonella* serovars, including *S.* Gallinarum and *S.* Pullorum and research on this topic is continuously developing (Kisiela *et al.*, 2005; Jeon *et al.*, 2007; Batista *et al.*, 2016; Ren *et al.*, 2017; Xiong *et al.*, 2017). Currently, modern diagnostic tools, such as real-time PCR, have become accessible to researchers and diagnostic laboratories. However, the design of specific probes and primers for this technique is a limitation as it depends on availability of genome sequences and bioinformatics analysis. The present work shows an extensive analysis and validation of a real-time PCR, using all its data, including the melting curve for differentiation of each PCR product by the respective *Tm*, and consequently for specific diagnosis of each biovar. As shown in Table 1, the sequences of the three primers and the length of each amplicon obtained have different *Tm* when the melting curve is evaluated, allowing differentiation of the serovar *Salmonella enterica* serovar Gallinarum from 2659 other serovars (Issenhuth-Jeanjean *et al.*, 2014), and also the differentiation between the biovars *S.* Gallinarum and *S.* Pullorum, which share large homologous genome regions (Batista *et al.*, 2015).

The applicability of this reaction by different laboratories worldwide depends on the costs of the reaction. Thus, we considered all the available DNA fluorescent dyes available and opted to use SYBr Green instead of probes. The use of the SYBr Green system in multiplex PCR in real time has been reported in different studies to identify pathogens, allergens, microbiological violations in contaminated food and antibiotic-resistant genes (Fukushima *et al.*, 2003; Ponchel *et al.*, 2003;

Varga & James, 2005; Liu *et al.*, 2006; Fan *et al.*, 2007; Pafundo *et al.*, 2010; Rajtak *et al.*, 2011; Kagkli *et al.*, 2012; Cheng *et al.*, 2013; Gomes *et al.*, 2014; Singh & Mustapha, 2014). However, this fluorescent dye, differently from probe systems, binds indistinctly to double-stranded DNA, emitting interfering levels of fluorescence in case of nonspecific amplification. Despite the *in silico* validation of the primers showing a specific site of annealing, the multiplex qPCR in real time described herein was developed after extensive titration of the primer concentrations and validation of annealing temperatures, to avoid nonspecific amplifications and primer dimers.

All three primer pairs used in the study were tested and validated with control strains, to determine the optimal concentration for each primer and the appropriate annealing temperature, in order to reach amplification and prevent the occurrence of non-specific reactions. Based on the results obtained with individually tested primers the multiplex qPCR in real time was performed. The optimized annealing temperature for this reaction was set at 63°C, which offered good conditions for the annealing of primers and amplification of the DNA, without the occurrence of any nonspecific fluorescence. The results obtained from the standardization of individual primer pairs or multiplex qPCR in real time were checked by agarose gel electrophoresis to confirm correct amplification based on the length of the products and the absence of nonspecific reactions as shown in Figures 1 and 2, respectively. The results of the multiplex qPCR in real time with positive control DNA samples are shown in Figure 3.

For diagnosis of each biovar, one amplification curve is obtained by the multiplex qPCR in real time. However, the differentiation is further performed by the analysis of the melting curve, obtained after amplification. This post-analysis shows the dissociation *Tm* of each amplicon, which is represented by a peak of fluorescence loss, when approximately 50% of double-stranded DNA (dsDNA) from amplicons is dissociated at the corresponding temperature and the SYBr Green reaction is lost. The evaluation of the *Tm* has shown consistency and sensitivity for the purpose of differential diagnosis of bacteria and virus with low mutation rates. Two factors influence the *Tm* of each amplicon: the length of the dsDNA fragment and the CG content. The higher values of these components require higher *Tm* for dissociation. The differential diagnosis between biovars in the qPCR in real time was performed using the *Tm* values. As shown in Figure 3(A) and 3(B) it is possible to notice two different dissociation peaks, corresponding to two different *Tm* in the melting curve after the multiplex PCR in real time. The first dissociation peak, with *Tm* of 78°C, refers to the amplicon with 97 bp obtained with primer pSGP, present exclusively in biovars *S.* Gallinarum and *S.* Pullorum (serovar *S.* Gallinarum), but not detected in any other *Salmonella* serovar. In the same multiplex qPCR also occurred the amplification for differentiation between biovars. As demonstrated in Figure 3(A), biovar *S.* Gallinarum is specifically detected by the primer pSG and the corresponding dissociation peak occurs at the *Tm* of 86.2°C, while the amplicon obtained with primers pSP designed for biovar *S.* Pullorum has a dissociation *Tm* of 84.8°C (Figure 3(B)). As carried out with the individual reactions, the efficacy, specificity and quality of the multiplex qPCR in real time were evaluated and confirmed by agarose gel electrophoresis, and the results are shown in Figure 2. The amplicons with 273 bp corresponded to biovar *S.* Gallinarum and with 260 bp corresponded to *S.* Pullorum.

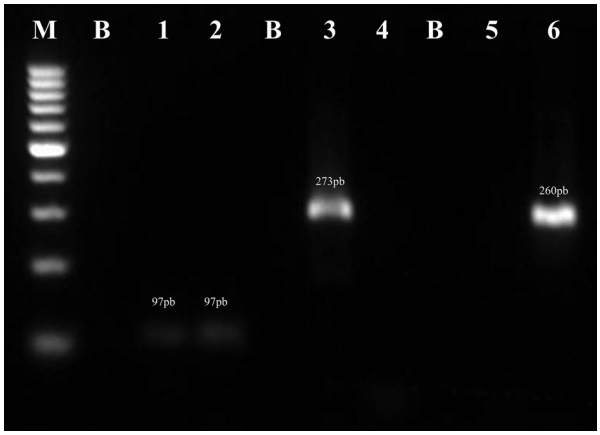

Figure 1. Agarose gel electrophoresis of the real-time qPCR amplicons using primer pairs separately. (M) Molecular Marker (100 bp); (B) Negative control (blank); (1) Primer pSGP with DNA of *S.* Gallinarum; (2) Primer pSGP with DNA of *S.* Pullorum; (3) Primer pSG with DNA of *S.* Gallinarum; (4) Primer pSG with DNA of *S.* Pullorum; (5) Primer pSP with DNA of *S.* Gallinarum; (6) Primer pSP with DNA of *S.* Pullorum.

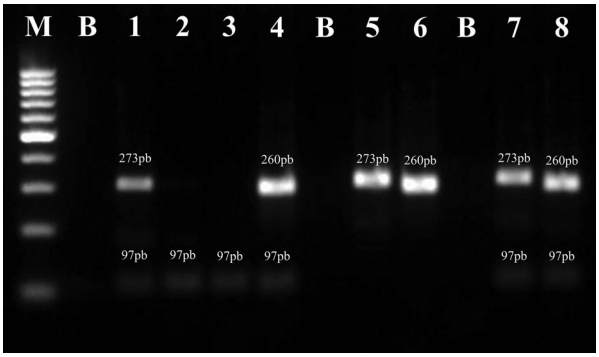

Figure 2. Agarose gel electrophoresis with amplicons from the multiplex qPCR in real time. (M) Molecular Marker (100 bp); (B) Negative control (blank); (1) Primers pSGP and pSG with *S.* Gallinarum DNA sample; (2) Primers pSGP and pSG with *S.* Pullorum DNA sample; (3) Primers pSGP and pSP with *S.* Gallinarum DNA sample; (4) Primers pSGP and pSP with *S.* Pullorum DNA sample; (5) Primers pSG and pSP with *S.* Gallinarum DNA sample; (6) Primers pSG and pSP with *S.* Pullorum DNA sample; (7) Primers pSGP, pSG and pSP with *S.* Gallinarum DNA sample; (8) Primers pSGP, pSG and pSP with *S.* Pullorum DNA sample.

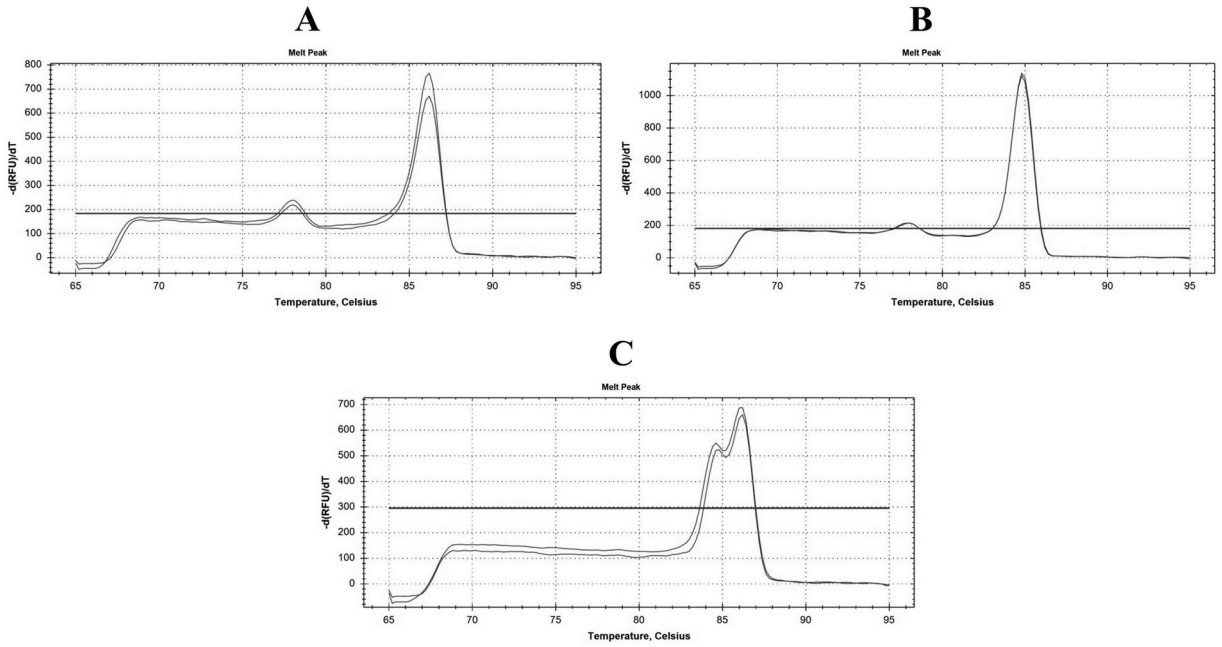

Figure 3. Melting curves and *Tm* peaks of the multiplex qPCR in real time for differential diagnosis between *S*. Gallinarum and *S*. Pullorum. (A) *Tm* peaks refer to the amplification of *S*. Gallinarum DNA samples with primers pSGP and pSG; (B) *Tm* peaks refer to the amplification of *S*. Pullorum DNA samples with primers pSGP and pSP; (C) *Tm* peaks refer to negative results using *S*. Enteritidis DNA samples in which no amplification with pSGP is noted (Tm 78°C), and amplifications with pSG, pSP occur together.

As shown in Figure 2, the duplex qPCR in real time can identify the pathogen at the level of the serovar and the biovar. However, differential diagnosis was achieved by the addition of the third primer pair composing the multiplex reaction, as per results shown in lanes 7 and 8. The differential diagnosis of these two typhoidal biovars has been previously reported by conventional PCR and methods (Ribeiro *et al*., 2009; Batista *et al*., 2013). However, the described techniques require two separate reactions and agarose gel electrophoresis for biovar differentiation, which affects time and costs for this reaction. In a single reaction the multiplex reaction was able to perform the identification and differentiation between the biovars, based on the specific *Tm* of the amplicons.

The specificity of the multiplex qPCR in real time for differential diagnosis between biovars was tested with DNA samples from non-typhoidal *Salmonella*. In such cases it is possible to notice the amplicons obtained with primers pSG (273 bp) or pSP (260 bp). However, the amplicon with 97 bp and *Tm* of 78°C is not present in non-typhoidal *Salmonella*. Thus, for differential diagnosis between these two typhoidal *Salmonella* biovars, it is necessary to include the primer pSGP for the differentiation from other non-typhoidal serovars. Moreover, as shown in Figure 3(C), amplification only with primers pSG and pSP may be used for diagnosis of other serovar from genus *Salmonella*, such as *S*. Enteritidis in cases when the pSGP amplicon with 97 bp (*Tm* peak of 78°C) is absent.

In addition to the amplification with positive diagnosis obtained with conventional PCR, another advantage of diagnosing with PCR in real time is the capacity of quantification of the subject of study. Based on the amplification data plotted, the cycle threshold (Cq) values are informative of the genetic material quantity, corresponding to the bacterial numbers in the sample. In the present study the multiplex qPCR in real time was developed to determine the sensitivity limit for quantification of *Salmonella*. The standard curve with genomic and plasmid DNA containing the genes of interest for each biovar was prepared. The conventional quantification of *Salmonella* by microbiological culture has a detection limit of 10^2 CFU/ml and lower bacterial numbers may not be detected without sample enrichment (Malorny *et al*., 2008). As shown in Figure 4, the standard curve was prepared with serially diluted DNA, and each dilution showed an interval of approximately four cycles for *S*. Gallinarum and three cycles for *S*. Pullorum (Table 2), corresponding to the dilution sequence of each biovar. Moreover, it was possible to detect and quantify all DNA samples, including the lowest dilution, corresponding to 10^1 CFU/ml from both biovars, demonstrating the high sensitivity obtained with the described reaction. Considering the high sensitivity, this diagnostic tool is capable of detecting low numbers of this pathogen, even without enrichment of the sample, consequently reducing time to obtain results.

Poultry production is often affected by acute and subclinical infections caused by enterobacteriaceae of genus *Salmonella* spp. and these bacteria are responsible for health impacts and large economic losses worldwide (Dobhal *et al*., 2014). The outcome of the disease depends on different factors, such as age of birds, genetic resistance and immunosuppressive

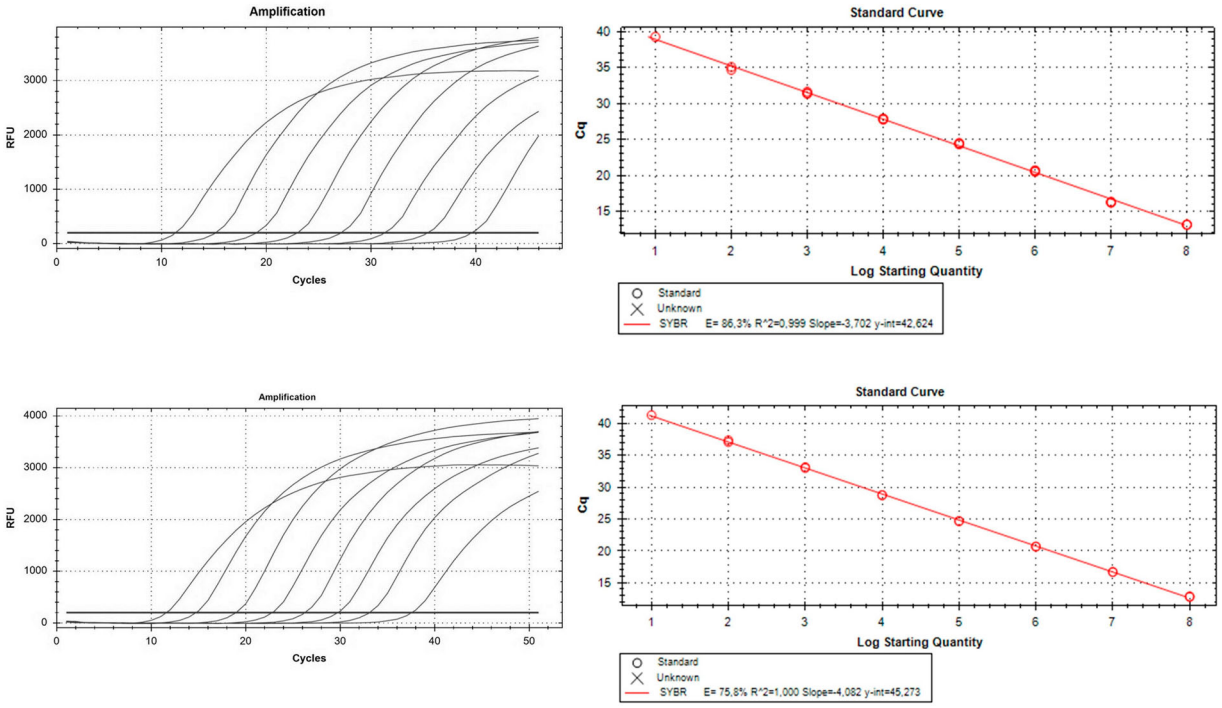

Figure 4. Amplification curves of serially diluted DNA using SYBr Green I (left) and quantitative standard curve (right) obtained by multiplex qPCR in real time for differential diagnosis and absolute quantification of *S*. Gallinarum and *S*. Pullorum. (A) *S*. Gallinarum DNA sample; (B) *S*. Pullorum DNA sample.

conditions. The option for treatment or elimination of the flock depends on the serovar. Thus, correct and fast diagnosis is very important for the right decision. Currently, the available techniques used for diagnosis have limitations, considering the time for results and low sensitivity to reduced bacterial loads in organ or environmental samples. Therefore, the proposed multiplex qPCR in real time has shown the capacity to diagnose the two avian host-specific *Salmonella* biovars in a single reaction. Additionally, a large number of non-typhoidal serovars can be detected in the reaction, differentiating from *S*. Gallinarum and *S*. Pullorum. Furthermore, the described technique is also capable of quantifying unknown bacterial loads, in addition to the positive or negative results obtained with conventional methods.

Table 2. Mean of amplification cycles (Cq) for each serial dilution of multiplex real-time qPCR to differential diagnosis and absolute quantification of *S*. Gallinarum and *S*. Pullorum.

		Mean of amplification cycles (Cq)	
Quantification point	CFU/ml[a]	*S*. Gallinarum	*S*. Pullorum
1	10^1	39.9	37.5
2	10^2	35.3	33.2
3	10^3	31.3	29.8
4	10^4	27.2	26.2
5	10^5	23.0	22.8
6	10^6	19.1	19.1
7	10^7	15.2	14.8
8	10^8	11.4	11.6

[a]CFU/ml: Colony forming units per ml.

Overall, the present study demonstrated the possibility of using a low-cost DNA dye such as SYBr Green in a convenient multiplex qPCR in real time for identification and quantification of two major typhoidal *Salmonella* biovars, capable of infecting birds and causing systemic infection and mortality. Thus, the described technique may facilitate the diagnosis and accelerate protocols to control the spread of these pathogens, which are of worldwide occurrence and which are able to disseminate both vertically and horizontally among avian hosts.

Disclosure statement

No potential conflict of interest was reported by the authors.

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