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Comparison of two affordable DNA extraction methods for molecular detection of *Salmonella* isolates from broiler farm's boot swabs

Comparação de dois métodos de purificação de DNA de baixo custo para a detecção molecular de *Salmonella* em isolados de propés de aviários

Comparación de dos métodos asequibles de extracción de ADN para la detección molecular de cepas de *Salmonella* aisladas de calzas de celulosa de granjas de pollos de engorde

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Abstract
There are many extraction methods available to purify bacterial DNA. PCR efficiency can vary depending on the quantity and quality of the template DNA used. This study evaluated the efficiency, quality, cost and rapidness of two in-house protocols, silica particles and Chelex-100 resin, for the extraction of twenty *Salmonella* isolates from boot swabs. The aim of this experiment was to compare the extraction methods for *Salmonella* spp. detection. DNA extraction was performed for each method and subjected to real-time PCR amplification. The amounts and integrity of DNA were determined using spectrophotometry and agarose gel electrophoresis, and the efficiency measured with real-time PCR. Limit of Detection (LOD) was determined with serial dilutions of a S. Typhimurium reference strain resulting in linear regression coefficients of R^2=0.992 (efficiency 119.31) for silica and R^2=0.958 (efficiency 93.33) for Chelex, with LOD of 10^{-4} for both. Silica particles method resulted in higher DNA yield, 85.01 ± 59.11 compared to 50.74 ± 12.95 and 260/280 ratio, 1.77 ± 0.02 versus 1.63 ± 0.13. DNA integrity was superior using silica, gel showed a unique band higher than 2.000 bp, while Chelex-100 was imperceptive or degraded. Regarding PCR results, the mean quantification cycle (*Cq*) for silica was 17.08 ± 0.73 and Chelex-100, 17.64 ±0.56 (suggesting lower DNA values). Results showed that both methods were effective for the DNA extraction of *Salmonella*, once PCR resulted positive for all samples, efficiency was higher for silica. Chelex-100 resin was performed in less time at a lower cost.
Keywords: Real-time PCR; Silica particles; Chelex-100 resin; *Salmonella.*

Resumo

Existem muitos métodos de extração para purificar DNA bacteriano. A eficiência da PCR pode variar dependendo da quantidade e qualidade do DNA alvo utilizado. Este estudo avaliou a eficiência, qualidade, custo e rapidez de dois protocolos, sílica e Chelex-100, para a extração de vinte isolados de *Salmonella* oriundos de propés. O objetivo do experimento foi comparar os métodos de extração para detecção de espécies de *Salmonella*. O DNA foi extraído e submetido à PCR em tempo-real. Determinou-se a quantidade e integridade do DNA por espectrofotometria e eletroforese em gel de agarose e eficiência por qPCR. Para calcular o limite de detecção (LOD) uma cepa de referência *S*. Typhimurium foi diluída em série resultando nos coeficientes de regressão linear de R^2=0,992 (eficiencia 119,31) e R^2=0,958 (eficiencia 93,33) para sílica e Chelex, com LOD de 10^{-4} para ambos. A Sílica rendeu mais DNA, 85,01 ± 59,11 comparado a 50,74 ± 12,95 e a razão 260/280 foi 1,77 ± 0,02 versus 1,63 ± 0,13. A sílica apresentou melhor integridade, gel com banda superior a 2.000 pb, enquanto o DNA do Chelex-100 pareceu imperceptível ou degradado. Com relação à PCR, a média do ciclo de quantificação (*Cq*) para sílica foi: 17,08 ± 0,73 e Chelex-100: 17,64 ±0,56 (sugerindo menor concentração de DNA). Os resultados mostraram que os métodos foram efetivos para a extração de *Salmonella*, uma vez que a PCR resultou positiva para todas as amostras, com eficiência superior para a sílica. O método Chelex-100 foi mais rápido e econômico.

Palavras-chave: PCR em tempo real; Sílica; Chelex-100; *Salmonella.*

Resumen

Existen muchos métodos para purificar ADN bacteriano. La eficiencia de la PCR puede variar dependiendo de la cantidad y cualidad de ADN utilizada. Este estudio evaluó la eficiencia, calidad, costo y rapidez de dos protocolos, sílica y Chelex-100 para la extracción de veinte aislados de *Salmonella* provenientes de calzas de celulosa. El objetivo del trabajo fue comparar los métodos de extracción para la detección de especies de *Salmonella*. ADN fue extraído y sometido a PCR en tiempo real. La cantidad e integridad fue determinada por espectrofotometría y electroforesis en gel de agarosa y eficiencia por qPCR. Para calcular el límite de detección (LOD), una cepa de referencia *S*. Typhimurium fue diluida en serie resultando en coeficientes de regresión lineal de R^2=0,992 (eficiência 119,31) y R^2=0,958 (eficiência 93,33) para sílica y Chelex, con el mismo LOD de 10^{-4}. La sílica resultó en mayor cantidad de ADN, 85,01 ± 59,11 comparada a 50,74 ± 12,95 y para la razón 260/280 1,77 ± 0,02 versus 1,63 ± 0,13. La integridad de la sílica pareció mejor en el gel, con una banda superior a 2.000 pb, ADN de Chelex-100 pareció imperceptible o degradado. La media del ciclo de cuantificación (*Cq*) de la sílica fue 17,08 ± 0,73 y del Chelex-100, 17,64 ±0,56 (sugiriendo menor concentración de ADN). Los resultados demostraron que los dos métodos fueron capaces de detectar *Salmonella*, una vez que la PCR fue positiva para todas las muestras, la eficiencia resultó superior para sílica. Chelex-100 fue más rápido y asequible.

Palabras clave: PCR en tiempo real; Sílica; Chelex-100; *Salmonella.*

1. Introduction

The *Salmonella* genus is divided into two species: *Salmonella enterica* and *bongori* (Wattiau et al., 2011). *Salmonella enterica*, one of world's most important foodborne pathogens, can be classified into different subspecies: enterica, salamae, arizonae, diarizonae, houtenae and indica (Kipper et al, 2022), and more than 2,650 serovars (Issenhuth-Jeanjean et al., 2014). Although many animals serve as *Salmonella* spp reservoirs, poultry and based products are included among the primary sources of salmonellosis in humans (Khan et al., 2022). Some *Salmonella* serovars are also associated with specific poultry diseases with very important economic losses (Shivaprasad, 2000).

Serotyping is the gold standard method used to identify different serovars but has a limited discriminatory power, requires specialized skills and reagents, and is restricted to reference laboratories (Hashemi & Baghbani-Arani, 2015). Molecular typing can be an alternative to identify types of *Salmonella* spp. (Haddad et al., 2001) due to its rapidness, consistency, reliability and reproducibility (Baratto et al., 2012). PCR methods have been used for the identification of many *Salmonella enterica* serovars, as Typhimurium, Enteritidis, Gallinarum and Pullorum (Rubio et al., 2019; Lee et al., 2009; Burgarel et al., 2011; Xiong et al., 2017). Although PCR has been successfully used for molecular typing (Liu et al., 2003; Borah et al., 2017; Salehi et al., 2011), its success can be challenged by the type of sample and quality of the DNA extraction method (Wilson, 1997).

DNA extraction consists in the chemical or physical separation of DNA from other cell components through techniques which include organic, non-organic or adsorption methods (Gupta, 2019). Silica particles and Chelex-100 resin are non-organic

methods. DNA extraction protocols are very important for the success of molecular techniques. Extracted DNA purity is an important issue in the sensitivity and usefulness of PCR for infectious pathogens (Hossain-Ripon et al., 2011).

Finding a suitable DNA isolation system to satisfy your *downstream* application needs is vital for the successful completion of the agent detection. Thus, the aim of this experiment was to compare two in-house DNA purification methods, silica particles and Chelex-100 resin, for the detection of culture isolates of *Salmonella spp.* from boot swabs. The amounts and integrity of DNA were determined using spectrophotometry and agarose gel electrophoresis, time and costs were also calculated. To assess the suitability of the extracted DNA for performing molecular techniques, the recovered DNA was processed by a Probe-based real-time PCR and a standard curve was constructed.

2. Methodology

The study was conducted in the Bacteriology and Molecular Biology Laboratories of Marcos Enrietti Diagnostic Center, Curitiba, Paraná, Brazil with laboratory's routine samples and a *S.* Typhimurium reference strain (ATCC 14028).

Samples Selection

Boot swabs from routine surveillance processed at the laboratory were used to recover and detect *Salmonella* positive samples in broiler farms from the West region of Parana State, Brazil. A reference strain of *S.* Typhimurium was used to generate a standard curve and calculate the limit of detection (LOD) for each extraction method.

Bacterial Isolation

First, samples were submitted to a pre-enrichment step in buffered peptone water (BPW) at 35 - 37 ºC for 24 hours. Then the samples were processed to isolate *Salmonella* using the inoculation in two enrichment broths. Two milliliters (mL) of BPW in 20 mL of tetrathionate (Merck, Darmstadt, GE) with novobiocin (TT), iodine and bright green (Inlab, São Paulo, BR) and 0.2 mL of BPW in 20 mL of Rappaport-Vassiliadis Soya (RVS) (Merck, Darmstadt, GE), incubated at 42 - 43 ºC for 24 hours. The enrichment broth was then plated on two selective agars, brilliant green agar (BGA) and xylose lysine dextrose (XLD) selective media (Merck, Darmstadt, GE) and incubated at 35 - 37 ºC for 18 - 24 hours. Suspected *Salmonella* colonies (pinkish white on BGA and black colonies on XLD) were submitted to triple sugar iron agar (TSI) (Merck, Darmstadt, GE), sulfur iron motility agar (SIM) (Acumedia Lab, Heywood, UK), lysine iron agar (LIA) (Merck, Darmstadt, GE), and urea broth incubation. Colonies suspected of being *Salmonella* spp. isolated in nutrient agar were submitted to further tests. A conventional slide agglutination test was used for screening, with poly O antiserum (SSI Diagnostica A/S, Hillerod, DK) for the identification of *Salmonella* spp. serotypes, according to the Ministry of Agriculture, livestock and food supply (Mapa) Normative instruction (NI) number 126, from November 3rd 1995, which establishes the procedures for *Salmonella* spp. isolation.

Salmonella Isolates

Twenty *Salmonella* isolates were included in this study. *Salmonella* colonies were identified on BGA and XLD plates, serotyped and posteriorly collected. Isolated samples were diluted in DEPC water to final concentration of 2 - 5 colonies/mL. After thoroughly homogenization, aliquots of 1 mL were placed in microtubes and frozen at - 80 °C until DNA extraction by either silica particles or Chelex-100 resin. For each procedure they were further organized in duplicate aliquots of 200 uL per method. DNA extraction rounds included a negative control for each 5 samples extracted to identify possible cross contamination among samples. If negative control amplified, the extraction round shoul be repeated, no amplifications were observed.

Standard Curve, Limit of Detection (LOD), Costs and Time of extraction method completion

A ten-fold dilution series (pure, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of *S.* Typhimurium DNA (ATCC 14028) extracted by Chelex-100 resin and Silica particles were used to generate a standard curve and for the limit of detection (LOD). A linear regression analysis was performed by plotting the quantification cycle (*Cq*) values against the logarithm of the copy number of the gene targeted. Limit of detection was considered the lowest concentration at which 95% of the positive samples were detected with the real-time PCR (Bustin et al., 2009). For each procedure the frozen cultures were organized in two aliquots of 200 uL per method, A and B, extracted by each method and then diluted tem-fold in DEPC water (Applied Biosystems™, San Diego, CA, USA) for posterior use in real-time PCR. The time spent to finish the extraction using each of the two methods was estimated with a chronometer (Kasvi®, Curitiba, PR, BR), time spent for solution preparation was not included. The cost was based on chemical reagents and plastics (tubes and pipette tips) and calculated in U.S. dollars.

DNA Extraction with Chelex-100 resin

Salmonella isolates and the reference strain were thawed and homogenized. A 200 uL volume of 5 % solution of Chelex-100 resin (Sigma-Aldrich, St Louis, MO, USA) was added and then heated to 95 °C for 10 minutes and centrifuged at 4,500 g per 10 minutes, then 100 uL of the supernatant was recovered and transferred to an identified new tube (Lamballerie, Zandotti, Vignoli, Bollet & Micco, 1992).

DNA Extraction with Silica Particles

Salmonella isolates and the reference strain were thawed and homogenized. A 500 uL volume of L6 solution and 30 uL of Silica hydrated (Sigma-Aldrich, St Louis, MO, USA) were added and then homogenized in vortex, at room temperature for 30 minutes, after that, centrifuged at 12,000 g per 15 seconds, and the supernatant disposed. The silica pellet was washed twice with L2 buffer, twice with ethanol 70 % and once with acetone. The pellets were set to dry with open lids at 56 °C for 10 minutes. The elution was performed using 100 µL TE buffer (Tris 100 µM, EDTA 50 µM), vortexed and incubated at 56 °C for 15 minutes. Tubes were vortexed again and centrifuged at 12,000 g per 5 minutes and the supernatant, containing DNA/RNA, was transferred to a fresh tube and used for the real-time PCR. This method was an adaptation of a previous protocol (Boom et al., 1990). Reagents preparation is described in the supplementary material.

DNA concentration and purity

Ratio of absorbance at 230, 260 and 280 nanometers (nm) were used to assess DNA concentrations and purity with a Nanodrop (Life Technologies, Carlsbad, CA, USA) and the samples were stored at - 20 °C before real-time PCR amplification.

Bacterial DNA Integrity

DNA integrity was assessed by 0.5 and 1 % agarose gel electrophoresis. Five microliters of extracted DNA from each sample were treated with 1 uL of RNase solution (LGC Biotecnologia, Cotia, SP, BR) at 37 ºC for 30 minutes and then two microliters of Trackit loading buffer (Applied Biosystems™, San Diego, CA, USA) with 0.5 % gel red (Biotium. Inc., Hayward, CA, USA) were mixed in a 0.2 mL tube, placed in agarose gel and subjected to a voltage of 90 volts during 60 minutes using 1 X TBE running buffer (Tris-borate-EDTA) in an horizontal system (Loccus®, Cotia, SP, BR). Bands were observed under ultraviolet light and photo documented.

Molecular detection of *Salmonella* spp. in boot swabs

The extracted DNA from cultured strains of clinical isolates was subjected to real-time PCR using the kit *Salmonella* spp. (NovaGuatá, Curitiba, PR, BR), primers and a TaqMan probe with the fluorescence FAM, specific to detect *Salmonella* genus. The primer and probe sequences have not been released by the company. The 10 uL reaction volume used for each sample contained 6 uL of MasterMix, 3 uL of ultrapure DNase-RNase-free distilled water and 2 uL of extracted DNA. Kit's positive and negative controls were used in the same reaction volumes but with 1 uL of template, following the kit's instructions. The real-time PCR was performed using a 7500 Fast Real-time PCR System (Applied Biosystem, Waltham, MA, USA) in 0.2 mL thin-wall strips or 96 well plates (Thermo Fisher Scientific, Waltham, MA, USA) with the cycling parameters: 95 ºC for 10 minutes followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute.

For standard curves and LOD, with *S.* Typhimurium DNA, the kit *Salmonella* Typhimurium (NovaGuatá, Curitiba, PR, BR) was used, primers and a TaqMan probe with the fluorescence FAM, with primers and probe sequences not shared by the company. Reaction volume, cycle and conditions were the same as for *Salmonella* spp.

Data analysis

Real-time PCR results, measured in *Cq*, were analyzed using one-way ANOVA, P values < 0.05 were considered statistically significant. Statistical analysis was performed using Minitab Statistical Software, version 21.1.0.

3. Results

***Salmonella* isolates**

The serotypes isolated from boot swabs were *S.* Heidelberg and *S.* Minnesota, 4 and 16 samples respectively. Isolates number 13, 15, 16 and 17 were identified as *S.* Heidelberg, while all the others were *S.* Minnesota (Table 1). The DNA concentration and absorbance 260 nm and 280 nm ratio of extracted pure culture bacteria using the two extraction methods and DNA quantity of each one of the 20 positive isolates from boot swabs extracted in duplicate (A and B) are in Table 1.

Table 1 - Quantity, quality and real-time PCR results for isolates of *Salmonella* spp. extracted by Silica particles and Chelex-100 resin.

Isolate	Silica Quantity			Silica Quality			Silica qPCR			Chelex-100 Quantity			Chelex-100 Quality			Chelex-100 qPCR		
	A (ng/uL)	B (ng/uL)	Mean ± SD	A (260/280)	B (260/280)	Mean ± SD	A *Cq*	B *Cq*	Mean ± SD	A (ng/uL)	B (ng/uL)	Mean ± SD	A (260/280)	B (260/280)	Mean ± SD	A *Cq*	B *Cq*	Mean ± SD
1*	50.70	43.10	46.90 ± 5.37	1.76	1.84	1.80 ± 0.06	15.27	15.81	15.54 ± 0.38	48.70	102.10	75.40 ±37.76	1.82	1.86	1.84 ±0.03	15.95	15.30	15.63± 0.46
2*	37.50	50.50	44.00 ± 9.19	1.65	1.58	1.62 ± 0.05	15.80	15.13	15.47 ± 0.47	37.20	33.20	35.20 ± 2.83	1.62	1.75	1.69±0.09	16.75	17.78	17.27± 0.73
3*	49.70	47.60	48.65 ± 1.48	1.65	1.77	1.71 ± 0.08	18.82	17.31	18.07 ± 1.07	40.30	45.80	43.05 ± 3.89	1.67	1.67	1.67±0.00	16.37	16.77	16.57± 0.28
4*	32.40	31.10	31.75 ± 0.92	1.49	1.47	1.48 ± 0.01	17.48	16.42	16.95 ± 0.75	15.70	17.00	16.35 ± 0.92	1.58	1.68	1.63±0.07	20.77	19.42	20.10± 0.95
5*	70.80	48.20	59.50 ± 15.98	1.86	1.81	1.84 ± 0.04	17.72	16.46	17.09 ± 0.89	60.20	48.60	54.40 ± 8.20	1.75	1.80	1.78±0.04	15.13	14.94	15.04± 0.13
6*	93.10	53.60	73.35 ± 27.93	1.40	1.68	1.54 ± 0.20	17.21	16.27	16.74 ± 0.66	44.50	69.10	56.80 ± 17.39	1.73	1.68	1.71±0.04	14.60	15.79	15.20± 0.84
7*	120.90	45.60	83.25 ± 53.25	1.39	1.51	1.45 ± 0.08	17.91	17.28	17.60 ± 0.45	14.90	19.40	17.15 ± 3.18	1.67	1.67	1.67 ±0.00	20.99	20.33	20.66± 0.47
8*	123.60	64.90	94.25 ± 41.51	1.49	1.83	1.66 ± 0.24	17.14	16.78	16.96 ± 0.25	60.90	78.30	69.60 ± 12.30	1.86	1.91	1.89±0.04	16.70	17.28	16.99± 0.41
9*	181.80	133.10	157.45 ± 34.44	1.40	1.81	1.61 ± 0.29	18.16	16.62	17.39 ± 1.09	137.40	87.00	112.20 ± 35.6	1.92	1.90	1.91±0.01	13.84	16.02	14.93± 1.54
10*	374.80	30.60	202.70 ± 243.40	1.36	1.48	1.42 ± 0.08	18.02	19.04	18.53 ± 0.72	19.00	25.00	22.00 ± 4.24	1.62	1.62	1.62±0.00	20.64	19.06	19.85± 1.12
11*	25.50	68.40	46.95 ± 30.33	1.83	1.53	1.68 ± 0.21	17.43	15.33	16.38 ± 1.48	47.10	31.40	39.25 ± 11.10	1.66	1.69	1.68±0.02	19.62	18.06	18.84± 1.10
12*	50.80	117.10	83.95 ± 46.88	1.63	1.48	1.56 ± 0.11	16.23	15.42	15.83 ± 0.57	31.00	33.10	32.05 ± 1.48	1.68	1.68	1.68 ±0.00	19.17	19.24	19.21± 0.05
13**	70.80	174.30	122.55 ± 73.19	1.86	1.57	1.72 ± 0.21	15.32	15.69	15.51 ± 0.26	68.50	100.00	84.25 ± 22.27	1.84	1.85	1.85 ±0.01	17.40	19.44	18.42± 1.44
14*	66.40	193.30	129.85 ± 89.73	1.75	1.52	1.64 ± 0.16	14.67	14.90	14.79 ± 0.16	52.60	61.30	56.95 ± 6.15	1.75	1.77	1.76±0.10	18.27	15.71	16.99± 1.8
15**	12.70	319.70	166.20 ± 217.10	1.66	1.37	1.52 ± 0.21	19.42	22.10	20.76 ± 1.90	22.70	22.20	22.45 ± 0.35	1.76	1.86	1.81±0.07	18.97	18.04	18.51± 0.66
16**	34.00	35.00	34.50 ± 0.71	1.90	1.91	1.91 ± 0.01	17.36	16.61	16.99 ± 0.53	44.30	47.70	46.00 ± 2.40	1.79	1.82	1.81±0.02	18.06	17.44	17.75± 0.44
17**	237.80	23.10	130.45 ± 151.80	1.40	1.68	1.54 ± 0.20	18.33	20.15	19.24 ± 1.29	24.00	23.10	23.55 ± 0.64	1.95	1.96	1.96±0.01	18.42	18.68	18.55± 0.18
18*	81.70	48.20	64.95 ± 23.69	1.92	2.04	1.98 ± 0.08	16.71	14.41	15.56 ± 1.63	216.50	91.70	154.10 ± 88.25	1.72	1.73	1.73±0.01	15.65	15.96	15.81± 0.22
19*	156.90	10.10	83.50 ± 103.80	1.37	1.88	1.63 ± 0.36	22.02	19.24	20.63 ± 1.97	10.90	10.90	10.90 ± 0.00	1.86	2.00	1.93±0.10	20.53	20.10	20.32± 0.30
20*	66.50	50.00	58.25 ± 11.67	1.76	1.81	1.79 ± 0.04	15.19	16.23	15.71 ± 0.74	43.30	43.10	43.20 ± 0.14	1.82	1.82	1.82±0.00	15.23	17.08	16.16± 1.31
	Total		88.15 ± 59.11			1.63 ± 0.13			17.08 ± 0.73			50.74 ± 12.95			1.77±0.02			17.64± 0.56

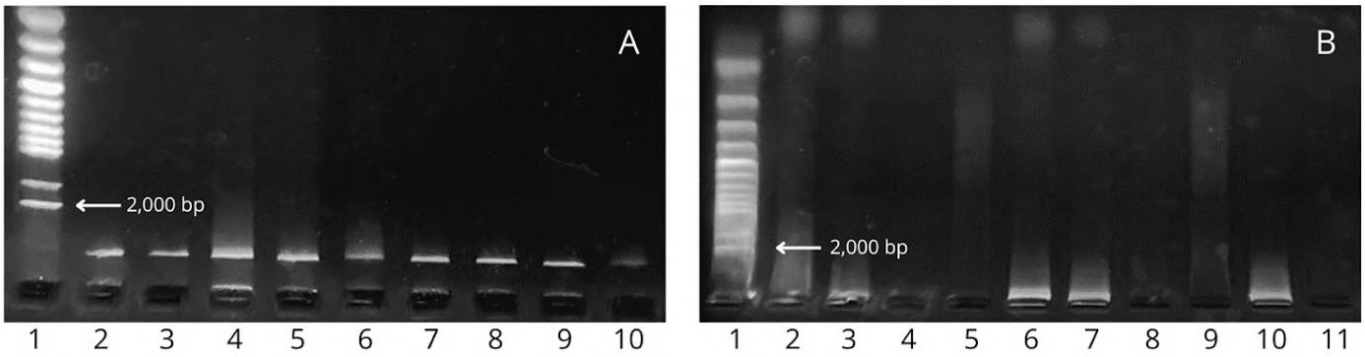
A and B represent the two aliquots of isolates extracted in duplicate. *Minnesota isolates. **Heildberg isolates. SD: Standard Deviation. *Cq*: Quantification Cycle. Source: Authors (2022).

The table shows that DNA quantity of each one of the 20 positive isolates from boot swabs extracted in duplicate (A and B) ranged from 10.10 to 374.8 ng/uL. The method with silica particles gave higher DNA yield, silica average for the 20 isolates was 88.15 and for Chelex-100 resin 50.74 nanograms per microliter. The 260/280 ratio varied from 1.36 to 2.04, silica particles average was 1.63 and Chelex-100 resin 1.77, a difference of 7.9% (Table 1). The standard deviation was 0.13 versus 0.02, for Silica and Chelex-100 resin, respectively. Even though the use of silica particles yielded a higher quantity of total DNA, compared to Chelex-100 resin, the 260/280 ratio was better, closer to 1.8, for the Chelex method.

Qualitative Analysis

The gel electrophoresis pattern of extracted *Salmonella* can be seen in figure 1. DNA intact high molecular weights were observed for samples extracted by Silica particles method (Figure 1.A).

Figure 1 - DNA integrity observation by agarose gel electrophoresis. A) *Salmonella* isolates DNA samples from boot swabs extracted by the Silica particles method. Lane 1: 100 bp Molecular Mass Marker (Ludwig, RS, BR). Lanes 2-10: Single band DNA > 2,000 bp samples extracted by Silica particles method. B) *Salmonella* isolates DNA samples from boot swabs extracted by the Chelex-100 resin method. Lane 1: 100 bp Molecular Mass Marker (Ludwig, RS, BR). Lanes 2-3, 6-7 and 10: DNA degraded, samples extracted by Chelex-100 resin method. Lanes 4-5, 8-9 and 11: imperceptive DNA, samples extracted by Chelex-100 resin method.



Source: Authors (2022).

Note that the DNA integrity was highly superior when using silica particles, the gel showed a unique band higher than 2,000 base pair (bp) while Chelex-100 resin was degraded or imperceptive, which could be seen as small fragments or sheared DNA.

Real-time PCR, Standard Curves and LOD

The *Cq* values of the probe-based real-time PCR presented the same detection power when the Silica or the Chelex-100 resin method were used, Table 1 (isolates) and figure 2 (ATCC bacterium). Regarding PCR, quantification cycle (*Cq*), it varied from 13.84 to 22.02, the average for silica was 17.08 and Chelex-100 resin 17.64 (higher values suggest lower DNA concentration), a percentage difference of 0.44 cycles, results were not statistically significant ($P > 0.05$), the standard deviation (SD) for the silica method was 0.73 versus 0.56 % for Chelex-100 resin (Table 1).

S. Typhimurium DNA (ATCC 14028) was extracted by Silica and Chelex-100 resin. A ten-fold serial dilutions of DNA recovered by each method was performed and then submitted to real-time PCR. Results can be observed in Table 2.

Table 2 - Ten-fold serial dilution of reference strain *S.* Typhimurium DNA (ATCC 14028) extracted by Silica and Chelex-100 resin, quantification cycle (*Cq*) and Mean.

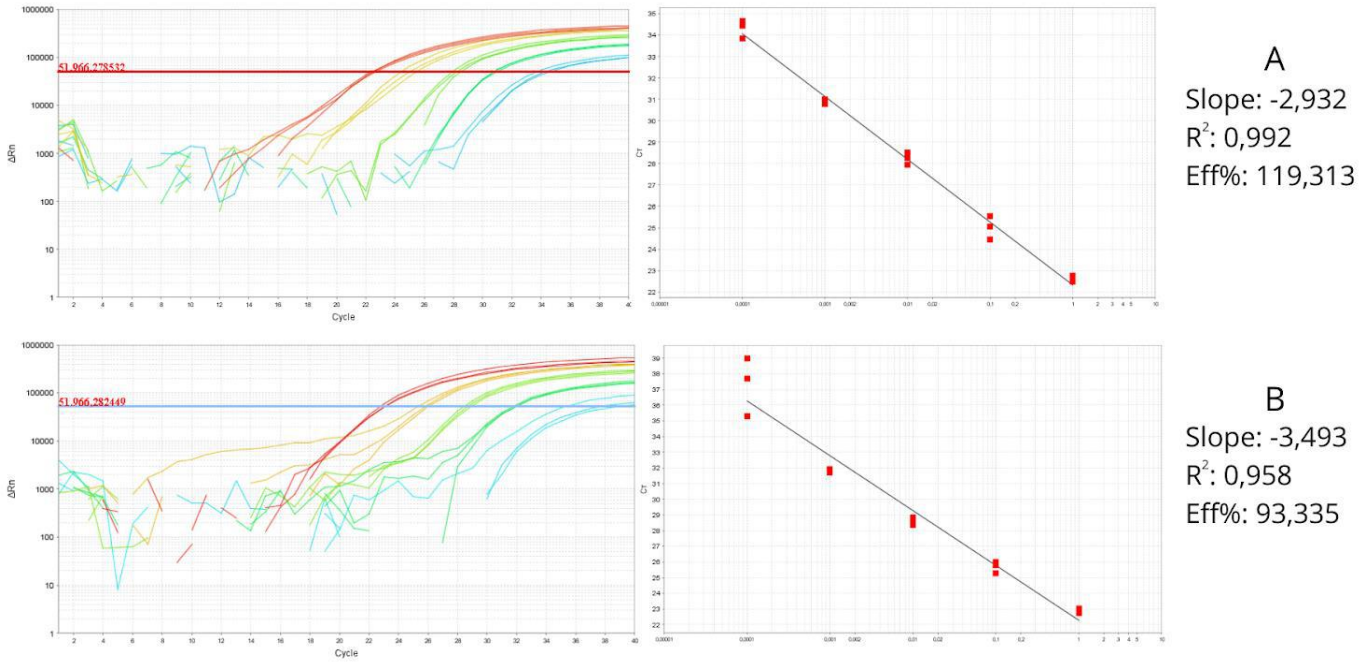
DNA Concentration	**Silica Particles (*Cq*)**			**Mean**	**Chelex-100 resin (*Cq*)**			**Mean**
1	22.52	22.48	22.76	22.59	22.72	23.03	23.03	22.93
10^{-1}	24.45	25.53	25.04	25.01	26	25.77	25.77	25.85
10^{-2}	27.95	28.27	28.53	28.25	28.84	28.63	28.37	28.61
10^{-3}	30.8	30.83	31.02	30.88	31.9	31.68	37.87	33.82
10^{-4}	33.84	34.67	34.43	34.43	35.29	38.98	37.69	37.32
10^{-5}	Und*	Und*	Und*	Und*	Und*	Und*	Und*	Und*
Cq Mean Value from 1 to 10^{-4}				27.80				28.21

Cq: Quantification Cycle. * Und: Undetermined. Source: Authors (2022).

When ten-fold serial dilutions of reference strain *S.* Typhimurium were analyzed in triplicate, the limit of detection for both methods was 10^{-4}. The average *Cq* value for Chelex-100 resin was 28.21 versus 27.80 for silica particles (Table 2), the difference was not significant ($P > 0.05$).

A Standard curve was performed for the *S.* Typhimurium DNA extracted with the Silica Particles method and Chelex-100 resin method (Figure 2).

Figure 2 - Standard curve with the correlation coefficient (R^2) resultant from plotting the quantification cycle (*Cq*) and the total DNA from *Salmonella* Typhimurium reference strain (ATCC 14028) after probe-based real-time PCR. A) Ten-fold serial dilution of *S.* Typhimurium DNA extracted from Silica Particles method: 1 to 1 x 10^{-4} and standard curve, efficiency (Slope and R^2). B) Ten-fold serial dilution of *S.* Typhimurium DNA extracted from Chelex-100 resin method: 1 to 1 x 10^{-4} and standard curve efficiency (Slope and R^2).



Source: Authors (2022).

The figure above shows the standard curve correlation coefficient (R^2) for the *S.* Typhimurium DNA extracted with the Silica Particles method of 0.992, the efficiency 119.31% and slope - 2.932. For the Ten-fold serial dilution of *S.* Typhimurium DNA extracted with the Chelex-100 resin method, R^2 resulted in 0.958, efficiency 93.33 and slope -3.493. The ideal correlation coeficiente should be one or ate least 0.99, so sílica was superior (Larionov et al., 2005).

Analysis of Costs and time spent for the two extraction methods

Regarding time and cost per sample, Chelex-100 resin was performed in less time at a lower cost. Time spent for silica extraction of a single sample was 01h 18m 27s versus 33m 04s, for Chelex-100 resin. When considering the costs of extraction per one sample, it was necessary to spend 1.06 versus 0.23 U.S. dollars for Silica and Chelex-100 resin, respectively.

4. Discussion

A total of 16 isolates of *Salmonella* Minnesota and 4 isolates of *Salmonella* Heidelberg, recovered from naturally infected boot swabs, were submitted to DNA extraction by two different in-house methods, silica particles and Chelex-100 resin, for the purpose of identifying the better-quality DNA. Methods were compared based on quantity, quality, integrity and suitability for real-time PCR purposes. Costs and time were also evaluated.

The silica particles method resulted in more DNA, on the other hand DNA quality evaluated by spectrophotometry was better for Chelex-100 resin. The amounts of DNA measured showed that the silica was able to recover more DNA, the average amount of nucleic acid recovered was 43% higher for silica (88.15 ng/uL x 50.74 ng/uL). The duplicated DNA extractions produced dissimilar DNA yields with standard deviations ranging from 0.71 to 217.10 for silica and 0 to 88.25 for Chelex-100 resin. Quality assessed by spectrophotometry resulted in average 1.63 x 1.77, for silica and Chelex-100 resin respectively, a ratio close to 1.8 is generally accepted as pure for DNA (Lucena-Aguilar et al., 2016), suggesting that Chelex-100 resin was better at purifying DNA from proteins. The integrity of the DNA recovered also varied depending on the method applied, Chelex-100 resin caused some shearing DNA (Figure 1.B), visible at the agarose gel, while silica resulted in a unique band (Figure 1.A). Regarding the C_q values obtained with *Salmonella* spp. TaqMan real-time PCR and the suitability of the extracted DNA for PCR amplification, both extraction methods resulted in sufficient amounts of template DNA for real-time PCR. All isolates resulted positive for both extraction methods using the molecular assay. Silica *Cq* mean value was smaller, 17.08, when compared with Chelex-100 resin, 17.64, but values did not differ statistically ($P > 0.05$). More copies of template DNA mean smaller number of amplification cycles needed to reach the quantification threshold (Walker, 2002), lower *Cq* value indicates a higher concentration of DNA; the difference of 0.56 cycles is consistent with the amounts of DNA, 43% more and 0.44 less cycles.

When comparing a standard curve for quantification of *S.* Typhimurium using either DNA recovered from silica or Chelex-100 resin, the qPCR reaction with DNA extracted by Silica particles method showed the lower mean *Cq* value, 27.8 compared to 28.21 for Chelex-100 resin, statistically non-significant ($P < 0.05$). The tenfold dilution evidenced a marked difference for higher dilutions, it seemed that when excess DNA was available, no differences were observed in PCR detection: pure, 10^{-1} and 10^{-2}, but when higher dilutions were observed: 10^{-3} and 10^{-4}, detection capacity was considerably lower for Chelex-100 resin: 30.88 x 33.82 and 34.43 x 37.32, respectively (2.94 and 2.89 cycles). The number of templates should double after each cycle (Booth et al., 2010), so the differences found at 10^{-3} and 10^{-4}, could indicate that DNA obtained by the silica method was almost eight times more concentrated than by Chelex-100 resin. Higher dilutions, 10^{-5}, could not be detected in any of the extraction methods used. Correlation coefficient (R^2), an efficiency measure, was better for silica: 0.992 (slope 119.31) than for Chelex: 0.958 (slope 93.33), ideally the values should be closer to one to reflect the linearity of the standard curve, 0.99 is well accepted (Larionov, Krause & Miller, 2005).

Regarding cost and time, Chelex-100 resin method was significantly more affordable and faster. Time spent for silica extraction of a single sample was 01h 18m 27s versus 33m 04s, for Chelex-100 resin, and cost 1.06 versus 0.23 U.S. dollars. Chelex-100 resin was 2.36 times faster and 4.6 times cheaper, so it could be an alternative method to explore when working with high yield DNA samples. The use of non-phenolic reagents as silica particles and Chelex-100 resin, can also be an alternative for phenolic compounds and chloroform methods, based on health-threatening chemicals, costly and unpleasant for laboratory workers (Karimnasab et al., 2013). Breakage of microorganisms with guanidine thiocyanate, as the sílica method used in this study, has been the standard initial step in genomic DNA extraction of microbial DNA for decades (Vingataramin & Frost, 2015). This approach is satisfactory for the extraction of gram-negative bactéria, like *Salmonella*. The ideal method for preparing DNA according to Lahiri, Bye, Nurnberger, Hodes and Crisp (1992) should be very rapid and economical, environmentally safe: preferably not requiring any organic solvents, produce good yield of high-quality DNA and flexible enough to be applied in frozen samples. Silica particles method and Chelex-100 resin are non-solvent DNA extraction methods, which apply to all these criteria. Nevertheless, alternative approaches for DNA extraction are required to prevent poor DNA recovery or the presence of co-extracted amplification inhibitors. Recognition of the exceptions that lead to insufficient DNA extraction efficiency is not possible without extensively studying and comparing methods.

5. Conclusion

This study compared two extraction methods to isolate high quality DNA to be efficiently amplified using real-time PCR. Chelex-100 resin was found to be a very simple, fast, affordable and cost-effective method. By the other hand, the method using silica particles was more efficient in isolating higher yields of high-quality DNA. This result indicates that for concentrated samples, high bacterial load, the two DNA extraction methods evaluated can detect isolates of *Salmonella* spp. from boot swabs.

Future studies should focus on diferente nucleic acid extraction methods for low bacterial load specimens and evaluate the efficiency of methods with scarce DNA.

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References

Baratto, C. M., Gelinski, J. M. L. N., Bernardi, A. Z., Marafon, A., & Braun, F. (2012). Potential use of molecular-typing methods for the identification and characterization of *Salmonella enterica* serotypes isolated in the poultry production chain. *Brazilian Journal of Poultry Science*, 14, 173-179.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M., & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *Journal of clinical microbiology*, *28*(3), 495-503.

Booth, C. S., Pienaar, E., Termaat, J. R., Whitney, S. E., Louw, T. M., & Viljoen, H. J. (2010). Efficiency of the Polymerase Chain Reaction. *Chemical Engineering Science*, 65(17), 4996-5006.

Borah, P., Porwollik, S., Desai, P., Nayak, P., Borah, P. P., Cheng, P., & McClelland, M. (2017). A simplified multiplex PCR-based typing method for common *Salmonella enterica* serovars supported by online server-based detection system. *The Indian Journal of Medical Research*, *146*(2), 272.

Brasil (1995). Ministério da Agricultura. Pecuária e Abastecimento. Portaria nº 126 de 03 de novembro de 1995. Normas de Credenciamento e Monitoramento de Laboratórios de Diagnóstico das Salmoneloses Aviárias (*S*. Enteritidis. *S*. Gallinarum. *S*. Pullorum e *S*. Typhimurium). Diário Oficial da União. Brasília. Seção 1. p. 3. Recuperado: 22/09/2022. Secretaria de Agricultura e Abastecimento. Coordenadoria de Defesa Agropecuária. https://www.defesa.agricultura.sp.gov.br/legislacoes/portaria-sda-126-de-03-11-1995.372.htm

Bugarel, M., Granier, S. A., Weill, F. X., Fach, P., & Brisabois, A. (2011). A multiplex real-time PCR assay targeting virulence and resistance genes in *Salmonella enterica* serotype Typhimurium. *BMC microbiology*, *11*(1), 1-11.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, 55(4), 611-622.

de Lamballerie, X., Zandotti, C., Vignoli, C., Bollet, C., & de Micco, P. (1992). A one-step microbial DNA extraction method using "Chelex 100" suitable for gene amplification. *Research in microbiology*, *143*(8), 785-790.

Gupta, N. (2019). DNA extraction and polymerase chain reaction. *Journal of cytology*, *36*(2).

Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M. F., Hughes, S. L., Inwald, J., Hewinson, R. G., & Durand, B. (2001). Spoligotype Diversity of *Mycobacterium bovis* Strains Isolated in France from 1979 to 2000. *Journal of Clinical Microbiology*, 39(10), 3623-3632.

Hashemi, A., & Baghbani-Arani, F. (2015). The effective differentiation of *Salmonella* isolates using four PCR-based typing methods. *Journal of applied microbiology*, *118*(6), 1530-1540.

Hossain-Ripon, M. K., Hasan, M., Ahasan, M. M., Alam, M. W., & Kabir, S. (2011). Comparison of three different methods of genomic DNA extraction from gram positive and gram negative bacteria. *Journal of Experimental Biosciences*, *2*(1), 55-60.

Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., de Pinna, E., Nair, S., Fields, P. I., & Weill, F. X. (2014). Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. *Research in microbiology*, *165*(7), 526-530.

Karimnasab, N., Tadayon, K., Khaki, P., Moradi Bidhendi, S., Ghaderi, R., Sekhavati, M., & Asadi, F. (2013). An optimized method from *Salmonella enterica* Enteritidis for PCR experiments. *Archives of Razi Institute*, *68*(2), 105-109.

Khan, A. S., Pierneef, R. E., Gonzalez-Escalona, N., Maguire, M., Li, C., Tyson, G. H., Ayers, S., Georges, K., Abebe, W., & Adesiyun, A. A. (2022). Molecular Characterization of *Salmonella* detected along the broiler production chain in Trinidad and Tobago. *Microorganisms*, *10*(3), 570.

Kipper, D., Mascitti, A. K., De Carli, S., Carneiro, A. M., Streck, A. F., Fonseca, A. S. K., Ikuta, N. & Lunge, V. R. (2022). Emergence, Dissemination and Antimicrobial Resistance of the Main Poultry-Associated *Salmonella* Serovars in Brazil. *Veterinary Sciences*, *9*(8), 405.

Lahiri, D. O., Bye, S., Nurnberger, J. I., Jr, Hodes, M. E., & Crisp, M. (1992). A non-organic and non-enzymatic extraction method gives higher yields of genomic DNA from whole-blood samples than do nine other methods tested. *Journal of biochemical and biophysical methods*, *25*(4), 193-205.

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, *6*(1), 62.

Lee, S. H., Jung, B. Y., Rayamahji, N., Lee, H. S., Jeon, W. J., Choi, K. S., Kweon, C. H. & Yoo, H. S. (2009). A multiplex real-time PCR for differential detection and quantification of S*almonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats. *Journal of Veterinary Science*, *10*(1), 43-51.

Liu, Y., Lee, M.-A., Ooi, E.-E., Mavis, Y., Tan, A.-L., & Quek, H.-H. (2003). Molecular Typing of *Salmonella enterica* Serovar Typhi Isolates from Various Countries in Asia by a Multiplex PCR Assay on Variable-Number Tandem Repeats. *Journal of Clinical Microbiology*, 41(9), 4388–4394.

Lucena-Aguilar, G., Sánchez-López, A. M., Barberán-Aceituno, C., Carrillo-Avila, J. A., López-Guerrero, J. A., & Aguilar-Quesada, R. (2016). DNA source selection for downstream applications based on DNA quality indicators analysis. *Biopreservation and Biobanking*, *14*(4), 264-270.

Minitab 21.1.0 Statistical Software (2022). [Computer software]. State College, PA: Minitab Inc. (www.minitab.com)

Rubio, M. S., Penha, R. A. C., Almeida, A. M., Barbosa, F. O., & Berchieri, A. (2019). Duplex Real-Time PCR Using Sybr Green I for Quantification and Differential Diagnosis between *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Brazilian Journal of Poultry Science*, *21*(1), 1-6.

Salehi, T. Z., Madadgar, O., Tadjbakhsh, H., Mahzounieh, M. R., & Feizabadi, M. M. (2011). A molecular study of the *Salmonella enterica* serovars Abortusovis, Typhimurium, and Enteritidis. *Turkish Journal of Veterinary & Animal Sciences*, *35*(5), 281-294.

Shivaprasad, H. L. (2000). Fowl typhoid and pullorum disease. *Revue scientifique et technique (International Office of Epizootics)*, *19*(2), 405-424.

Vingataramin, L., & Frost, E. H. (2015). A single protocol for extraction of gDNA from bacteria and yeast. *BioTechniques*, *58*(3), 120-125.
Walker, N. J. (2002). A Technique Whose Time Has Come. Science, *296*(5567), 557-59.

Wattiau, P., Boland, C., & Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Applied and environmental microbiology*, *77*(22), 7877-7885.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and environmental microbiology*, *63*(10), 3741-3751.

Xiong, D., Song, L., Tao, J., Zheng, H., Zhou, Z., Geng, S., Pan, Z., & Jiao, X. (2017). An Efficient Multiplex PCR-Based Assay as a Novel Tool for Accurate Inter-Serovar Discrimination of *Salmonella* Enteritidis, *S.* Pullorum/Gallinarum and *S*. Dublin. *Frontiers in microbiology*, *8*, 420.

SUPPLEMENTAL MATERIAL

Silica Solution

60g of silicon dioxide [(SiO_2). Sigma. USA] was suspended in 500 mL of ultrapure water and left to sediment for 24 hours at room temperature. Approximately 430 mL of supernatant were discarded and ultrapure water was added to a total volume of 500 mL and vigorously shaken to resuspend silica pellets. Another sedimentation step of 5 hours at room temperature took place and the same volume of supernatant was disposed of by suction. Finally, 600 µL of HCl were added to adjust pH. Aliquots of 2 mL were autoclaved for 20 minutes at 121°C and stored at 4°C.

L2 Solution

120g of guanidine thiocyanate (GuSCN) was added in 100 mL of 0.1 M Tris hydrochloride (pH 6.4) and heated at 60°C under continuous shaking.

L6 Solution

120g of guanidine thiocyanate (GuSCN) was added in 100 mL of 0.1 M Tris hydrochloride (pH 6.4). Subsequently, 22 mL of 0.2 M EDTA solution adjusted with NaOH to pH 8.0 and Triton X-100 were added and homogenized