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ANIMAL SCIENCE

Optimization of the molecular detection assay of *Salmonella* (MDS®) for drag swab samples of aviaries

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Abstract: Detection of *Salmonella* sp. is important for the broiler chicken production chain because it is one microorganisms involved in food-borne diseases. Thus, this study performed the optimization of a technique of Loop-mediated isothermal DNA amplification (LAMP) through the 3M™ Molecular Detection Assay 2: *Salmonella* (MDS®), in accordance with Ordinance number 126 of the Ministry of Agriculture, for the detection of *Salmonella* sp. in drag swab. The methodology followed ISO 16140-2: 2016, with the analysis naturally contaminated drag swab samples collected from broiler aviaries and artificially contaminated with salmonella ATCCs. Of the 300 samples processed in protocol A (pre-enrichment tetrathionate broth (TT)), 45 were positive for *Salmonella* sp., 242 negative, one false-positive, and 12 false-negative, while of the 300 samples analyzed in protocol B (pre-enrichment brain-heart infusion broth (BHI)), 40 were positive, 256 negative, one false-positive, and three false-negative. The result for protocol A was a sensitivity of 79%, specificity of 99.6%, Positive Predictive Value (PPV) of 98%, and Negative Predictive Value (NPV) of 95%; and for protocol B, 93% sensitivity, 99.6% specificity, 98% PPV, and 99% NPV. Both protocols were associated with the reference method (p>0.05), concluding that the MDS® can be used for the qualitative detection of *Salmonella* sp.

Key words: Bird contamination, molecular detection, pathogenic bacteria, poultry farming.

INTRODUCTION

Salmonella sp. is one of the main microorganisms involved in food-borne diseases (FBDs) (Shinohara et al. 2008, Borges et al. 2013). This enterobacteria is a Gram-negative rod, usually mobile, capable of producing acids and gases from glucose (Sterzo et al. 2008, Brasil 2011). Although present in the commensal microbiota of the gastrointestinal tract of animals and humans, it has great pathogenic potential, triggering the disease after migration via a phagocytic mononuclear system, where it infects and replicates in macrophages and from there spreads in the carrier's organism. *Salmonella* sp. assume a relevant role in health issues related to poultry farming, causing economic damage to the sector (Shinohara et al. 2008, Brasil 2011, Borges et al. 2013).

In 2010, food contaminated by chemical, physical, and/or biological agents was responsible for causing 600 million cases of FBDs in the World (WHO 2015). In Brazil, between 2000 and 2017, 12,503 outbreaks of FBDs were reporting, 3,196 of which were laboratory confirmed and 2,593 with identification of the etiological agent. Of the latter, 92.2% were caused by bacteria, of which *Salmonella* sp., *Escherichia coli*, and *Staphylococcus aureus* were prominent (Brasil 2018).

In this sense, the Ministério da Agricultura, Pecuária e Abatecimento (MAPA) established Normative Instruction (IN) number20, of October 21st, 2016 (Brasil 2016), which seeks the control and monitoring of *Salmonella* sp. in commercial poultry establishments and slaughterhouses as an alternative to decrease the contamination of poultry and provide greater protection to the consumer of chicken meat. This normative cites the obligation of microbiological analyzes before slaughter for preventing positive batches for *Salmonella* Typhimurium, *S.* Enteritidis, *S.* Pullorum, *S.* Gallinarum, monophasic *S.* (1,4[5],12:-:1,2), and monophasic S. (1,4[5],12:i:-) when they are wrongly slaughtered, without knowledge about the sanity of the batch, thus brings a safer food to the consumer's table.

The official assays for the detection of *Salmonella* sp. in Brazil are carried out according to Ordinance number 126 of November 3rd, 1995 of MAPA (Brasil 1995). Although the applied method is efficient and reliable, it is time-consuming and can last for a week (Freschi et al. 2005, Gandra et al. 2008, Possebon 2012). In the IN number 20 (Brasil 2016) authorizes the use of molecular detection methods to optimize this process.

The equipment 3M™ Molecular Detection Assay 2 – *Salmonella* (MDS®) was validated in November 2016 for using in food microbiological analyses and in February 2019 for using in animal health samples in accordance with EM ISO 16140-2:2016 (ISO 2016) to contribute to this streamlining of the *Salmonella* sp. qualitative detection process. For samples of primary (fecal) production, such as drag swab, it uses pre-enrichment protocols with Tetrathionate Broth (TT), supplemented with iodine solution and 0.1% brilliant green solution, a culture medium selective for the microorganism in question.

This project aimed to optimize a LAMP technique associated with bioluminescence by means of MDS equipment® in accordance with Ordinance number 126 (Brasil 1995). The hypothesis is that using brain-heart infusion broth (BHI) as a pre-enrichment protocol, a non-selective culture medium, can increase multiplication and favor the growth of *Salmonella* sp., increasing reliability, sensitivity, specificity of this technology, also providing a reduction in costs in the detection of the microorganism and a decrease in labor for carrying out the tests.

MATERIALS AND METHODS

Experiment period and stages

From July 2019 to December 2020, the equipment denominated 3M™ Molecular Detection Assay 2 – *Salmonella* (MDS®) was optimized. So, bringing it closer to Brazilian legislation, as shown in figure 1.

This research followed ISO 16140-2:2016 (2016) with modifications, composed of two stages: a comparative study of the results of naturally and artificially contaminated samples. They are obtaining by two pre-enrichment protocols, confirming them with a reference method, Ordinance number 126 (Brasil 1995).

The alternative protocols of pre-enrichment were calling Protocol A, with the use of selective broth culture medium tetrathionate (TT), according to the instructions of the equipment manufacturer, and protocol B, proposed in this research, with the use of non-selective brain-heart infusion broth (BHI).

Experiment locations and sampling

For the comparative study with naturally contaminated samples, 300 drag swab samples collected in duplicates, totaling 600 samples collected in aviaries in the Western state of Santa Catarina, Brazil. Samples were collecting over a 15-week period, 20 samples in duplicates per week. Samplings were conducted according

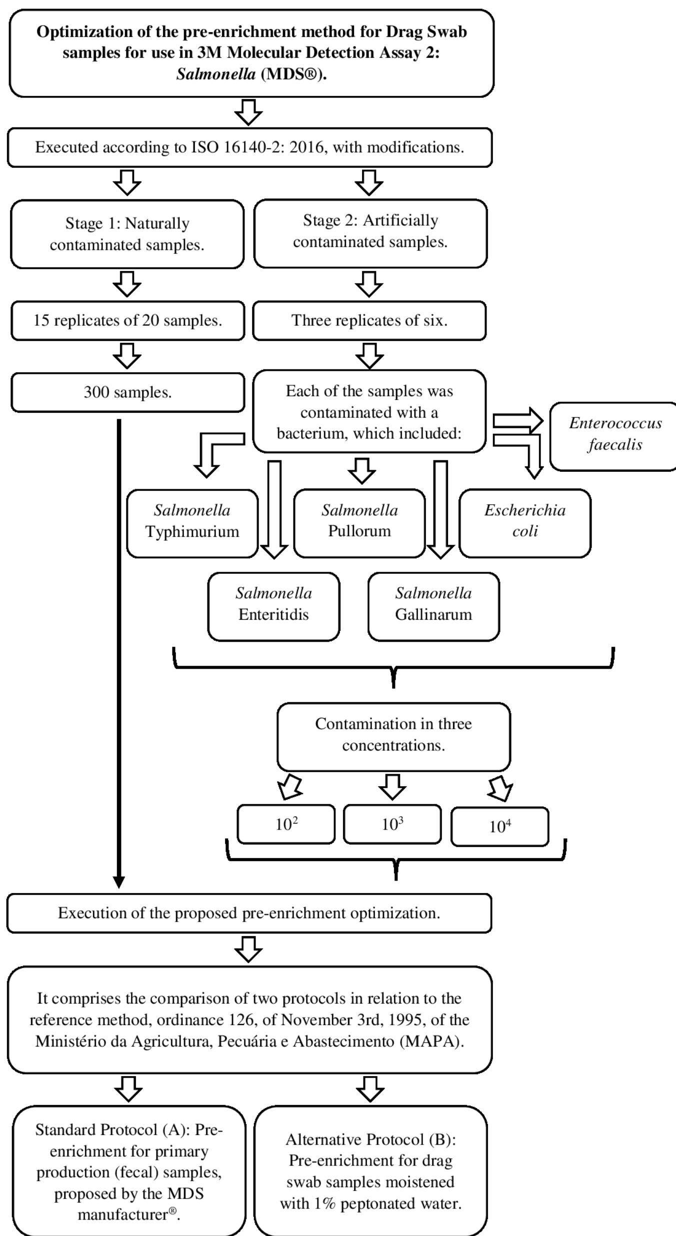

Figure 1. Flowchart of the optimization research development of the *Salmonella* (MDS®) molecular detection assay for drag swab samples from aviaries.

to IN number 20 of October 21st, 2016 (Brasil 2016), namely: 2 moistened drag swabs were used with peptonated water 1% (MERCK KGaA®) per aviary, each one covering 50% of the installation area. These were stored in an isothermal container and sent to the Laboratory of Animal Pathology and Bromatology to carry out the tests in a period of up to 48 hours. Each sample of the same aviary has been submitting to the reference protocol and to the alternative protocols.

Preparation of artificially contaminated samples

Drag swabs have been contaminated with standard strains of *Salmonella* Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076, *S.* Pullorum ATCC 13036, *S.* Gallinarum ATCC 9184, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212, at concentrations of 10^2, 10^3, 10^4 CFU/ml by means of the McFarland nephalometric scale (0.5 NTU corresponding to 1.5 x 10^8 CFU/

ml), subsequently proceeding to plating by depth and bacterial count on Soybean Tryptone Agar plate - TSA (MERCK KGaA®) performed in triplicate. This protocol has been developing in three replicates, totaling 108 samples processed.

Alternate pre-enrichment protocols for drag swab samples moistened with 1% peptonated water primary production

Initially, the samples have been submitting to the pre-enrichment protocols. Protocol A was carried out according to the orientation of the MDS manufacturer® for drag swab, 225 ml of TT broth (MERCK KGaA®), supplemented with Iodine solution (MERCK KgaA®) and 0.1% Brilliant Green solution (MERCK KgaA®), and incubated at a temperature of 37 °C, for 22 to 24 hours. The samples of Protocol B, proposed in this research, aims to approach the current legislation for isolation and detection of *Salmonella* sp. (Brasil 1995), was added with 100 ml of 1% peptonated water (MERCK KGaA®) and homogenized five times. Then, 2 ml of the sample has been transferring to a glass tube containing 20 ml of BHI broth (MERCK KGaA®). Later, the incubation has been carried out at 36 °C ±1 for 18 to 24 hours.

3M™ Molecular Detection Assay 2 – *Salmonella* (MDS®)

After the pre-enrichment stage (protocol A and B), 20 µl of the sample and 20 µl of sterile buffered peptonate water (MERCK KGaA®) have been transferring to the lysis solution tubes for the preparation of the negative and positive controls. The lysis tubes have been then taking to the heating block at 100 °C ± 1 for 15 minutes. Subsequently, they have been placing in the room temperature block for 5 minutes. Next, 20 µl of the sample and lysed controls have been transferring to the corresponding reagent tube. To complete the procedure, the tubes have been transferring to the fast loading tray. After 60 minutes, the MDS® issued the result.

Reference or confirmatory protocol

The methodology described by Ordinance number 126 of November 3rd, 1995, of MAPA (Brasil 1995) was followed as a reference protocol of *Salmonella* sp. isolation, which consisted in the pre-enrichment, as previously mentioned in both protocols A and B. Then, selective enrichment has been performing, adding 200 µl and 2 ml of the previous sample in tubes containing 20 ml of Rappaport Vassialidis Soya broth (MERCK KGaA®) and 20 ml of TT broth (MERCK KGaA®), respectively. Incubation has been at a temperature of 41 °C ± 0.5 for 18 to 24 hours. Subsequently, the insulation was striated with Brilliant Green Agar (MERCK KGaA®) and Hektoen Agar (MERCK KGaA®), incubating the plates for 18 to 24 hours at 36 °C ±1, then reading the characteristic colonies of *Salmonella* sp.

Subsequently, preliminary biochemistry tests have been striated (Triple Sugar-Iron Agar - TSI (Difco®), Lysine Iron Agar - LIA (Difco®), SIM Medium (MERCK KGaA®) , and Urea (MERCK KGaA®)) incubated at 36 °C ±1 for 18 to 24 hours, then reading the results. Complementary biochemistry tests (Phenylalanine deaminase (Sigma-Aldrich®), Simmons citrate (MERCK KGaA®), Malonate (Difco®) Methyl Red - MR (MERCK KGaA®), VP - Voges-Parker (MERCK KGaA®), Phenol Red Broth Control (MERCK KGaA®), Lactose (Synth®), Sucrose (InLab®), Maltose (InLab®), Mannitol (Synth®), Dulcitol (InLab®), Glucose (MERCK KGaA®), Decarboxylase Control (Sigma-Aldrich®), Lysine decarboxylase (Difco®), Ornithine decarboxylase (InLab®), Arginine dehydrolase (InLab®)) were also conducted, proceeding with incubation at 36 °C ±1 for 18 to 24 hours, then observing the reading.

The strains that presented preliminary and complementary biochemical profile compatible

with *Salmonella* sp. have been characterizing antigenically through the rapid agglutination test. Antisomatic serum “O” polyvalent of *Salmonella* sp. (SSI Diagnostics®) has been used in naturally contaminated samples; for artificially contaminated samples, polyvalent antisomatic serum “O “(SSI Diagnostica®), O:4 (SSI Diagnostics®), Hi (SSI Diagnostics®), and H: 2 (SSI Diagnostics®) has been used for *S.* Typhimurium; O:9 (diagnostic SSI®), Hg (SSI diagnosis®), Hm (SSI Diagnostics®), Hp (SSI Diagnostics®), Hq (SSI Diagnostics®) for *S.* Enteritidis; and O:4 (diagnostic SSI®), O:9 (SSI Diagnostics®) for *S.* Gallinarum and *S.* Pullorum.

Statistical analysis

Statistical analyses has been for naturally contaminated samples of aviaries. For the calculations the Fisher’s Exact Test, specificity, sensitivity, PPV e NPV the R software (R Core Team 2019) was used, with an interval of confidence of 95%, to express the efficiency of the results issued by the MDS®.

RESULTS

Naturally contaminated samples

Of the 300 samples processed in protocol A, 45 were positive for *Salmonella* sp., 242 negative, one false-positive, and 12 false-negative concerning the confirmatory methodology, Ordinance number 126 (Brasil 1995), while of the 300 samples analyzed in protocol B, 40 were positive, 256 negative, one false-positive, and three false-negative.

The sensitivity obtained by protocol A was of 79%, the specificity of 99.6%, the PPV of 98%, and the NPV of 95%; and protocol B obtained 93% sensitivity, 99.6% specificity, 98% PPV, and 99% NPV (Table I).

The Fisher’s Exact Test has showed that both protocols haven’t been (*p-value* > 0.05).

Artificially contaminated samples

In protocol A, 80.55% of the samples analyzed were positive by MDS® and 75% by the reference method. In protocol B, 100% of the samples were detecting. Otherwise, it is important to indicate that the samples not detected by the MDS® and those not detected by the reference method were contaminated with the standard strains of *Salmonella* Gallinarum ATCC 9184. Samples contaminated with the strains of *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were not detected by the MDS® method and traditional method in both protocols (A and B) (Table II).

DISCUSSION

Salmonella sp. is a bacterium of great relevance for industrial poultry farming as it is one of the causing agents of FBDs, indirectly causing great economic losses to the sector, as it can cause economic embargoes, bans on trade in the product imposed by importing countries

Table I. Results of pre-enrichment protocols A and B regarding sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) in relation to the reference method.

	PROTOCOL A	PROTOCOL B
Sensitivity	0.79 (0.66, 0.89)	0.93 (0.81, 0.99)
Specificity	0.99 (0.98, 1.00)	0.99 (0.98, 1.00)
PPV	0.98 (0.88, 1.00)	0.98 (0.87, 1.00)
NPV	0.95 (0.92, 0.98)	0.99 (0.97, 1.00)

95% interval of confidence.

(Shinohara et al. 2008, Borges et al. 2013). For this reason, two Brazilian legislations aim the control and monitoring of this bacterium in the poultry chain. Therefore, the optimization of the molecular detection assay for *Salmonella* (MDS®) for its use in drag swab samples in animal health, bringing it closer to current legislation to expedite the detection of this pathogen in Brazil.

The MDS® was validated for animal health in February 2019, by ISO 16140-2:2016 (2016), which approves its use with the pre-enrichment protocol A using TT broth with supplementation of iodine solution and brilliant green solution, incubation at 37 °C, for 22 to 24 hours since it is a selective culture medium that favors the growth of the microorganism to be detected. However, to approximate the methodology with the Brazilian current legislation, making an equipment suitable for its use in food agribusiness in the country, this research suggests the use of BHI broth (protocol B), in an incubation temperature of 36 °C for 18 to 24 hours.

The results found were 93% sensitivity in the detection of *Salmonella* sp. for protocol B and 79% for protocol A, with a numerical difference between them but no significant statistical difference. The specificity of both protocols was equal, 99.6%, corroborating with the result found by Notomi et al. (2015), who reports that the lamp technique has high specificity, a technique used by MDS®. In the joint analysis of the sensitivity and specificity parameters of protocols A and B, with the VPN of 95% and 99%, respectively, and the VPP of 98% in both situations, indicates,

Table II. Artificially contaminated samples in protocols A and B using an alternative method (Molecular Detection Assay 2) and reference method (Ordinance 126).

Microorganism	Dilution	FIRST REPLICATE				SECOND REPLICATE				THIRD REPLICATE			
		PROTOCOL A		PROTOCOL B		PROTOCOL A		PROTOCOL B		PROTOCOL A		PROTOCOL B	
		MDS®	Ref.	MDS®	Ref.	MDS®	Ref.	MDS®	Ref.	MDS®	Ref.	MDS®	Ref.
***Salmonella* Typhimurium ATCC 14028**	10^2	+	+	+	+	+	+	+	+	+	+	+	+
	10^3	+	+	+	+	+	+	+	+	+	+	+	+
	10^4	+	+	+	+	+	+	+	+	+	+	+	+
***Salmonella* Enteritidis ATCC 13076**	10^2	+	+	+	+	+	+	+	+	+	+	+	+
	10^3	+	+	+	+	+	+	+	+	+	+	+	+
	10^4	+	+	+	+	+	+	+	+	+	+	+	+
***Salmonella* Pullorum ATCC 13036**	10^2	+	+	+	+	+	+	+	+	+	+	+	+
	10^3	+	+	+	+	+	+	+	+	+	+	+	+
	10^4	+	+	+	+	+	+	+	+	+	+	+	+
***Salmonella* Gallinarum ATCC 9184**	10^2	-	-	+	+	-	-	+	+	-	-	+	+
	10^3	-	-	+	+	-	-	+	+	-	-	+	+
	10^4	+	-	+	+	+	-	+	+	-	-	+	+
***Escherichia coli* ATCC 25922**	10^2	-	-	-	-	-	-	-	-	-	-	-	-
	10^3	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
***Enterococcus faecalis* ATCC 29212**	10^2	-	-	-	-	-	-	-	-	-	-	-	-
	10^3	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-

Caption: Ref: Reference Method; + Positive Result; - Negative Result.

according to Thrusfield (2004), that both protocols have greater ability to detect the true negative, confirming the hypothesis that it would be a good screening method. Note that there was no statistical difference between the protocols, demonstrating that both have the same chances of obtaining the same results.

The protocols were associated with Ordinance number 126 (Brasil 1995) ($p > 0.05$). In other words, both protocols A and B obtained reliable results, confirmed by the reference methodology, with the data obtained by the exact Fischer test. These results corroborate the study conducted by Fortes et al. (2013), who performed a 3M™ MDS® validation to detect *Listeria* spp. with the collection of environmental sponges from meat, seafood, and dairy processing sites. These same authors performed the validation comparing with the standard microbiological method for microorganism isolation. The results showed no significant statistical difference between the methods.

The numerical difference found in the sensitivity between the pre-enrichment protocols A and B may have occurred due to the interference of the culture medium used, favoring protocol B since the alternate methodology of protocol A used the TT broth, selective culture medium for *Salmonella* sp. In parallel, the addition of brilliant green, suppresses Gram-positive bacteria (Knox et al. 1943, Jeffries 1959). However, using this culture medium as pre-enrichment may not favor the multiplication of the microorganism in question. A BHI medium was used for protocol B which is highly nutritious and non-selective (ANVISA 2004, ISO 11133:2014). This may have contributed to the growth of *Salmonella* sp., with sufficient CFU/ml for detection by MDS® obtained.

Corroborating this research is the study conducted by De Paiva et al. (2006) who compared pre-enrichment stages with buffered peptonate water and direct enrichment with TT broth and selenite-novobiocin broth from 25 stool samples artificially contaminated with strains of *S.* Enteritidis and *S.* Typhimurium, stored for 24 and 96 hours. In this work, the authors demonstrated a numerical difference but no statistical difference in storage for 24 hours, obtaining isolation of 13 samples in direct enrichment and 19 samples in pre-enrichment. However, there was a statistically significant difference in storage for 96 hours, obtaining isolation in direct enrichment of 11 samples and in pre-enrichment of 21 samples. These results indicate a greater recovery of bacteria when non-selective pre-enrichment was performed, favoring the multiplication of the microorganism, without their selection.

The detection limit proposed by the manufacturer of the MDS® is 103 CFU/ml of *Salmonella* sp. In this study, the equipment detected up to 10^2 Typhimurium, Enteritidis, and Pullorum serovars in Protocols A and B. However, at concentrations 10^2 and 10^3 (protocol A) Sorovar Gallinarum was not detected. Additionally, detection of the microorganism occurred by the equipment at 10^4 in two of the replicates. However, there was no isolation by the traditional method. For the other samples of *S.* Gallinarum, detection occurred in all concentrations (10^2, 10^3, and 10^4) in protocol B, also confirming the hypothesis that the BHI culture medium favors the growth and multiplication of microorganisms, including fastidious bacteria such as *S.* Gallinarum better than the TT broth, even if it is selective for the microorganism in question.

The results of the naturally and artificially contaminated samples affirm the efficiency of the equipment for its purpose. In this perspective, the reproducibility study conducted by Bird et al. (2016) consisted of 16 laboratories evaluating the reproducibility of the MDS® method for peanut

butter and uncooked meat samples, by different people in different environments, demonstrating the reproducibility of the method, as well as for our study demonstrates the ability to detect a true negative, which makes it ideal for use as a screening method for the pre-enrichment and A and B protocols.

Therefore with this study, it is concluded that both protocols, A with the use of TT broth and B with the use of BHI broth, are associated with the reference method, as well as confirming the optimization of the molecular detection assay with the use of pre-enrichment protocol B, allowing the use of the equipment as a screening method, due to its ease of use, faster results and approximation of current legislation.

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Author contributions

Maiara C. Titon designed the research project, performed the laboratory tests present in the research, wrote the main text of the manuscript and prepared the figures. Diogenes Dezen designed the research project, made important revisions to the main text and figures. Paulo M. de A. Costa designed the research project, performed the statistics present in the manuscript and made revisions to the main text. Douglas W. Rizzotto designed the research project, performed the laboratory tests present in the research and made revisions to the main text. Sidiane F. Castanha designed the research project, performed the laboratory tests present in the research and made revisions to the main text. Alessandra F. Millezi coordinated the research, designed the research project and made important revisions to the main text and figures.