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Artigo Original/ Original Article

Validation of PCR method for mycoplasma detection in the Yellow Fever-vaccine quality control

Validação de método de PCR para detecção de micoplasmas no controle de qualidade da vacina de Febre Amarela

RIALA6/1706

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Recebido: 17.03.2016 - Aceito para publicação: 06.09.2016

ABSTRACT

Among the vaccines produced by Bio-Manguinhos, a major centre for manufacturing the immunobiological products in Latin America**,** stands out the yellow fever (YF) vaccine**.** To guarantee the excellence and safety of the YF vaccine, the quality control tests has to be performed throughout its production. The World Health Organization (WHO) demands the producers to guarantee the absence of *Mycoplasma orale*, *M. pneumoniae*, *M. gallisepticum* and *M. synoviae* in the biological products. Mycoplasma is a fastidious microorganism, requiring about 35 days for attaining the conclusive culturing test. In this study PCR methods were selected for amplifying 16S rRNA gene fragments for detecting mycoplasma in the intermediate products of YF vaccine. This standardized methodology was specific and sensitive to detect the low concentrations of mycoplasma in spiked intermediary vaccine products**;** and the absence of unspecific amplification was also demonstrated. The detection rates ranged from 3.1 to 12.5 colony forming units and showed 100 % of sensitivity and specificity in the tested samples**.** The PCR protocol for detecting mycoplasmal DNA in YF vaccine was validated by analysing 286 samples. Bio-Manguinhos produces annually 10,000,000 YF vaccine doses**,** and this method has been successfully employed, complementing the traditional approach in the mycoplasma detection since 2008.

Keywords. mycoplasma, PCR, vaccine, yellow fever.

RESUMO

Dentre as vacinas produzidas por Bio-Manguinhos, um importante centro de produção de imunobiológicos da América Latina, destaca-se a vacina de febre amarela (FA) que é produzida em ovos embrionados. Para garantir a excelência e a segurança da vacina, testes de controle de qualidade são realizados durante a produção. A Organização Mundial de Saúde (OMS) exige dos produtores a ausência de *Mycoplasma orale*, *M. pneumoniae*, *M. gallisepticum* e *M. synoviae* em produtos biológicos. Micoplasmas são micro-organismos fastidiosos, sendo necessários 35 dias para que os testes de cultura sejam conclusivos. Neste estudo foram selecionados métodos de amplificação de fragmentos do gene 16S rRNA para detecção de micoplasmas em produtos intermediários da vacina de FA. Esta metodologia padronizada foi capaz de detectar baixas concentrações de micoplasmas nos produtos intermediários e a ausência de amplificação inespecífica foi demonstrada. O limite de detecção variou entre 3,1 e 12,5 unidades formadoras de colônia**;** e nas amostras testadas a sensibilidade e a especificidade foram de 100 %. O protocolo de PCR para detecção de micoplasmas na vacina foi validado pela análise de 286 amostras. Bio-Manguinhos produz 10.000.000 doses de vacina de febre amarela por ano e, desde 2008, este método tem sido empregado com sucesso, complementando-se a abordagem tradicional.

Palavras-chaves. micoplasmas, PCR, vacina, febre amarela.

INTRODUCTION

Yellow fever (YF) is an acute, viral, mosquito-borne haemorrhagic fever that affects the immunologically susceptible individuals. YF virus is endemic in the tropical and subtropical regions in Africa and South-America where 200,000 cases with 30,000 deaths are estimated annually[1]. A live attenuated YF vaccine is already attainable, and it is one of the most effective available vaccines which have been administered to more than 400 million people worldwide with minimal incidence of severe side effects[2]. Max Theiler and his associates at Rockefeller University created the YF virus vaccine 17D**,** after performing several tissue sub-culturing passages of the Asibi strain, isolated from a Ghanaian patient in 1927. In Brazil the first vaccines were tested in 1937**,** and its production was later standardised with 17DD sub-strain[3].

Since it was pre-qualification by World Health Organization (WHO) in 2001, more than 155 million of YF vaccine doses were produced by Bio-Manguinhos/Fiocruz, and they have been exported to more than seventy countries, through the Pan-American Health Organization (PAHO) and United Nations Children Fund (UNICEF). This vaccine has been produced in the specific pathogen-free (SPF) chicken embryos according to the guidelines established by WHO[4]. Following three days of inoculation and incubation under controlled temperature and moisture, the viable embryos are harvested, grinded and centrifuged. The resulting viral suspension is frozen with samples harvested for quality control testing. Following the quality control approval, the viral suspension is thawed, and diluted by adding the stabilizers, originating the bulk product. The formulated bulk is lyophilised in vials containing 5, 10 or 50 doses (**Figure 1**). Before the vaccine release, several quality control tests should be performed according to the WHO guidelines**.** The mycoplasma controlling devices in the YF vaccine intermediates, e.g. viral suspension and bulk, are mandatory[5].

Mycoplasmas are the smallest self-replicating bacteria, without a cell wall and require the sterols and fatty acids for providing the cytoplasm membrane stability, being taxonomically separated from other bacteria, and belonging to the Mollicutes class (mollis = soft; cutis = skin in Latin). Additionally, they present reduced genomes and biosynthetic capabilities. Infections in animals and humans are rarely fulminant - mycoplasmas usually live in harmony with their host. Mycoplasma researches and laboratory diagnosis performances have been hampered because of its fastidious cultivation features[6]. However, they might also accidentally infect the cell cultures. This contamination invalidate the results achieved from cell culture, and it is harmful for the diversity of biological products[7,8].

The mycoplasmal culturing takes about 35 days to provide the final result, and this work is particularly laborious and expensive. However, this procedure is a "Gold-Standard" methodology stated by WHO for performing mycoplasma testing in biological products, including YF vaccine and intermediates**.** Even though, the culture media formulations for mycoplasma detection are not described in the WHO requirements.

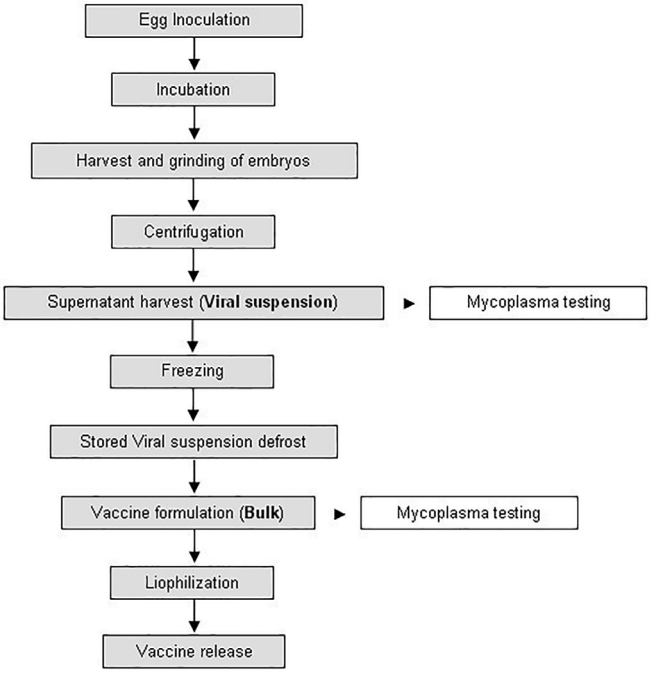

Figure 1. Flowchart of YF vaccine produced in Bio-Manguinhos. The words in bold and inside the parenthesis highlight the vaccine intermediate samples used in this study

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PCR-based and other alternative methods for mycoplasma detection have been considered for being applied to the biological products manufacture, and these techniques can also be used as long as they have been validated[5].

Method validation assures that a new analytical assay shows the true results confirming the procedure as "adequate to use"[9]. For validating an alternative qualitative molecular methodology, such as PCR, it is critical to determine the essential parameters of the assay[10]. Thus, the objective of this study was to standardize and to validate a rapid and accurate diagnostic PCR assay for detecting mycoplasma contamination in YF vaccine, by analyzing the vaccine intermediates, according to the WHO requirements.

MATERIAL AND METHODS

Bacterial Strains, Media and Growth Conditions

The following mycoplasma strains (all acquired from the American Type Culture Collection, ATCC) were used throughout this study: *M. gallisepticum* (ATCC 15302), *M. orale* (ATCC23714), *M. synoviae* (ATCC25204) and *M. pneumoniae* (ATCC 15492).

The medium used for mycoplasma growth was a variation of Modified Frey's Medium[11]. This medium, called as PPLO medium, is composed of 22.5 g/L mycoplasma base broth (Becton Dickinson, Phoenix, AZ, USA), 12 g/L glucose (Sigma Chemical Company, St Louis, MO, USA; 0.025 g/L phenol red (Sigma Chemical Company) and 20 % (v/v) supplement. This supplement is consisted of 62.5 g/L yeast extract (Becton Dickinson), 1.25 g/L thallium acetate (Sigma Chemical Company), $5x10^6$ U/L penicillin (Sigma Chemical Company), 0.625 g/L NAD (Sigma Chemical Company), 0.625 g/L cysteine-HCl (Sigma Chemical Company), 30 % (v/v) horse serum (Hyclone, Logan, UT, USA) and 30 % (v/v) swine serum (Hyclone). For solid medium, 12 g/L noble agar (Beckton Dickinson) were added. Before use, the horse and swine sera were inactivated at 56 °C for 30 minutes, and NAD and cysteine-HCl were mixed and incubated during 15 minutes at room temperature. The final pH was adjusted to 7.8.

The growth of mycoplasma strains was set up from the freeze-dried sample vials. Initially the growth was confirmed by the change of liquid media colour and production of the "fried egg"-shaped colonies observed under a stereomicroscope on solid media. From broth cultures, the strains were ten-fold diluted into 9 mL of broth medium from 10^{-1} to 10^{-5} and incubated at 37 °C in 5 % CO_2. *Mycoplasma orale* was grown in anaerobic conditions. As the mycoplasma growth does not cause turbidity, therefore it was detected by acidifying the broth medium, observing the color change from red to yellow (except for *M. orale*, in which the arginine is hydrolysed and the broth is alkalized).

Non-Mollicutes class microorganisms were also obtained from the ATCC by the selectivity tests, e.g., *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Clostridium sporogenes* (ATCC 11437), *Escherichia coli* (ATCC 8739), *Micrococcus luteus* (ATCC 9341), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 6538). These microorganisms were grown in the broth and solid soy casein medium (Becton Dickinson, Phoenix, AZ, USA) at 37 °C, except *C. Albicans* grown at 25 °C. All of these strains were cultivated in aerobiosis, excepted *C. sporogenes* (anaerobiosis).

Production and titration of mycoplasma seed lots

Forty mililiters of each fresh mycoplasma culture, i.e. before colour changing or after 96 hours-incubation, the suspensions were mixed with 40 mL of sterile medium and 20 mL of sterile glycerol PA (Merck, Darmstadt, Germany). One mL aliquots were stored in vials at -20 °C, generating the seed lot. Before and after storage, three of these 1 mL-vials were ten-fold diluted in liquid medium up to 10^{-9}, and 200 µL of each dilution were plated on the solid medium. The plates were incubated for seven days, and then they were observed in a stereomicroscope at 40 X magnification. After incubating for one week, the colonies were counted. Quantification of seed lots took into account only the plates

containing 50 to 300 colonies**,** and it was prepared from each reference strain upon its fifth passage. The determination was done by calculating the average among the replicates.

Seed lots of non-Mollicutes class microorganisms were also prepared and quantified in their specific medium as mycoplasma seed lots.

Sampling

The vaccine intermediates (viral suspension and bulk) were analyzed without (plain) and with the adding of microorganisms (spiked) at known concentrations by seed lots dilution.

DNA extraction

DNA extraction was carried out using the commercial Wizard SV Genomic DNA Purification System (Promega, Madison, USA) with 1 mL of each sample. The manufacturer protocol for DNA extraction from mouse tail tissue was followed, with slight modifications. The incubation at 55 °C for 16-18 hours was shortened to three hours. The suspension washings were done with 500 µL instead of 650 µL of washing buffer**,** and the DNA elution was performed in a single step after two minutes incubation with 50 µL of DNAse-free water.

Polymerase Chain Reaction (PCR)

Four primer sets were assessed for detecting mycoplasma by means of PCR technique (**Table**). The primer sets were conceived by following groups: van Kuppeveld et al[12], the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* – DSMZ[13], Bruchmuller et al[14], and the Japanese Pharmacopoeia[15]. Initially, the PCR techniques were set up with varied reagent molarities and cycling temperatures, i.e., following the protocols described by the diverse authors. After testing these protocols, the assays were carried out using those primers in the standard PCR conditions. Further tests were performed for optimizing the magnesium chloride concentration, the annealing temperature and the primer concentration. In order to minimize the deviations in methods comparison**,** the PCR standardization assays were set up with the same DNA samples.

The optimized protocol for PCR reaction comprised of 25 µL final volume of 200 µM of each dNTP (Invitrogen, San Diego, USA), 0.6 µM of each primer, 0.5 U Taq Go Flexi polymerase, enzyme buffer 1X, 2 mM $MgCl_2$ (Promega, Madison, WI, USA) and 5 µL of template DNA. This mix was heated at 95 ºC for 10 min, then cycled 30 times,

Table. List of synthetic DNA oligonucleotide primers used in this study

Primers Reference	Forward	Reverse	Amplicon size
12	5'actcctacgggaggcagcagta3'	5'tgcaccatctgtcactctgttaacctc3'	~700 bp
13	5'cgcctgagtagtacgttcgc3' 5'cgcctgagtagtacgtacgc3' 5'tgcctgagtagtacattcgc3' 5'tgcctgggtagtacattcgc3' 5'cgcctgggtagtacattcgc3' 5'cgcctgagtagtatgctcgc3'	5'gcggtgtgtacaagacccga3' 5'gcggtgtgtacaaaacccga3' 5'gcggtgtgtacaaaccccga3'	~500 bp
14	5'gagcaaacaggattagatac3'	5'accatgcaccayctgtcaytc3'	~300 bp
15	5'acaccatgggagytggtaat3' 5'gtgsggmtggatcacctcct3'	5'cttcwtcgacttycagacccaaggcat3' 5'gcatccaccawawacyctt3'	~400 bp

Footnote: The amplicons size may vary slightly among the mycoplasma species

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and submitted to the denaturation at 94 ºC for 30s; annealing at 55 ºC for 30s and extension at 72 ºC for 90s. The final extension took place at 72 °C for 7 minutes.

All of the primers used in this study were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA), and the amplifications were carried out in the thermocycler T3000 (Whatman Biometra, Goettingen, Germany). Following the DNA amplification, the results were analyzed on 1 % (w/v) agarose gels in 1X TAE buffer (40 mM Tris-acetate, 1mM EDTA). The electrophoresis was set up on 5V/cm. After the assay running, the gels were stained by incubating in 0.5µg/mL ethidium bromide solution for 10 min and distained in water for 30 min. Data were recorded by the Photodocumentation EDAS 290 System (Kodak, Rochester, NY, USA), under the UV light emission.

PCR Methodology Validation

PCR methodology validation was performed by running this assay and analysing 286 samples of vaccine intermediates and the saline solution with or without mycoplasma spiked with other microorganisms unrelated to the Mollicutes class. In order to improve the reliability, each replica of the same assay was set up by new spiked samples, i.e., diluted the microorganisms from a different seed lot vial performed at different day.

Selectivity

Seventeen samples of vaccine and its intermediates with 25 CFU were used, including pure vaccine and those spiked with each one of the four mycoplasma species, and also the microorganisms unrelated to the Mollicutes class diluted in saline solution. For this assay, 25 colony forming units (CFU) of each of the four mycoplasma species were employed. The used mycoplasma inoculum is close to the WHO requirements for liquid medium approval in growth promotion test. In order to evaluate whether higher amounts of unrelated microorganisms could produce false-positive results, they were diluted into 200 CFU per sample in saline solution. The assay acceptance criteria were (i) the observation of a band with roughly 500 bp in 1 % agarose gel for spiked mycoplasma samples and (ii) the absence of this band for samples spiked with *C. sporogenes*, *M. luteus, B. subtilis*, *S. aureus*, *C. albicans*, *E. coli* and *P. aeruginosa* as well as in non-spiked samples. This parameter was evaluated in two replicas, totalizing 34 samples.

Limit of detection (LOD)

Pure and spiked vaccine intermediates were used with different mycoplasma concentrations. The assay took place in duplicate for every intermediary product of each mycoplasma species and their respective controls (plain products), analyzing 112 samples. This assay aimed at determining the lowest amount of each mycoplasma, which could be detected in the YF-vaccine intermediates.

Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, Analytical Accuracy and Kappa Index

Samples of both vaccine intermediates were spiked with 30 CFU of each mycoplasma species. Each test was performed with five samples of every intermediary product, one plain and four spiked with each one of assayed mycoplasmas. This parameter was evaluated in 14 replicas, i.e., eight replicas performed by one operator and two replicas by each one of the three other different operators, totalizing 140 samples. The objective was to use these data in a contingency table, and the assay acceptance criteria achieving 100 % sensitivity, at least 95 % analytical accuracy and 0,61 of Kappa Index, i.e., no false-negative results and a strong agreement rate among the evaluated parameters.

RESULTS

Methods Comparison

Initially, the PCR efficiency was evaluated using each of the four primer sets as previously described by other authors[12-15]. The *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ) primers were used following the protocol previously described, but no amplicon was produced from the templates of YF vaccine intermediates spiked with mycoplasma.

Next, we tested the Japanese Pharmacopoeia methodology[15] and PCR targeted products were obtained in three of four analysed mycoplasma (e.g., *M. orale*, *M. synoviae* and *M. pneumoniae*) in the second round of amplification, both at 100 CFU and 10 CFU spiked samples. However, the absence of amplification in the material spiked with *M. gallisepticum* made this protocol unsuitable. The primers mentioned by van Kuppeveld et al[12] were tested using the described parameters. Like the Japanese Pharmacopoeia primers, the van Kuppeveld oligonucleotides yielded amplicons for three mycoplasma species, but not for *M. gallisepticum*. Finally, the Bruchmuller primer set[14] was tested, and no bands with the predicted sizes were produced. All of this initial comparison was performed with the same extracted DNA samples.

After testing the four protocols to detect mycoplasma targeted DNA (e.g., *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*), we concluded that none of the assays met the WHO criteria set up at the beginning of the present study.

Each of the four primers sets was tested in the standard PCR conditions, and using the same DNA samples extracted from 100 and 10 CFU of each mycoplasma diluted in the vaccine intermediates. Only the set described by DSMZ group[13] showed positive amplification of all of the species. For this reason, DSMZ primer set was used in several amplification reactions, differing in magnesium chloride concentration, primer molarity, and annealing temperature, to define an optimized protocol in order to improve the detection limit of the assay (data not shown). The best amplification efficiency was achieved with 2 mM magnesium chloride, 0.6 µM of each primer and at 55 °C annealing temperature. This modified protocol produced amplicons in all of the four tested mycoplasma species both in bulk and in spiked viral suspension with 100 CFU and 10 CFU (**Figure 2**). Therefore, this protocol was selected for conducting the method validation evaluation.

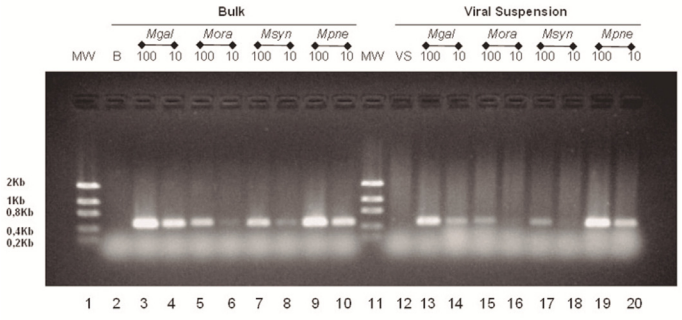

Figure 2. Electrophoretic analysis of amplicons produced by using optimized protocol employing DSMZ primers set. The whole volume of PCR product was used for performing electrophoretic analysis on 1 % agarose gel. Lane 1 and 11: Molecular weight marker "Low Mass DNA Ladder". Lane 2: Bulk negative control. Lanes 3-10: Bulk spiked with 100 and 10 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*, respectively. Lane 12: Viral suspension negative control. Lanes 13-20: Viral suspension spiked with 100 and 10 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*, respectively

Method Validation

Selectivity

In order to demonstrate the specificity of the primers used in the PCR reactions, we tested the plain and spiked samples of vaccine intermediates with each of the four mycoplasma species, as well as the quantified microorganisms unrelated to the Mollicutes class. The expected amplicon was obtained for all of the four tested mycoplasmas, spiked in both intermediary products (**Figure 3**, lanes 3 to 6 and 8 to 11). No amplification was detected for the negative controls of vaccine intermediates as expected (**Figure 3**, lanes 2 and 7).

Out of the seven bacterial and fungal species tested as unrelated microorganisms, none provided DNA amplification, determining the specificity of the primers and of the protocol employed for mycoplasma amplification (**Figure 3**, lanes 12 to 18). This assay was prepared in duplicate, for demonstrating the absence of unspecific amplification products both for pure vaccine intermediates and for unrelated microorganisms.

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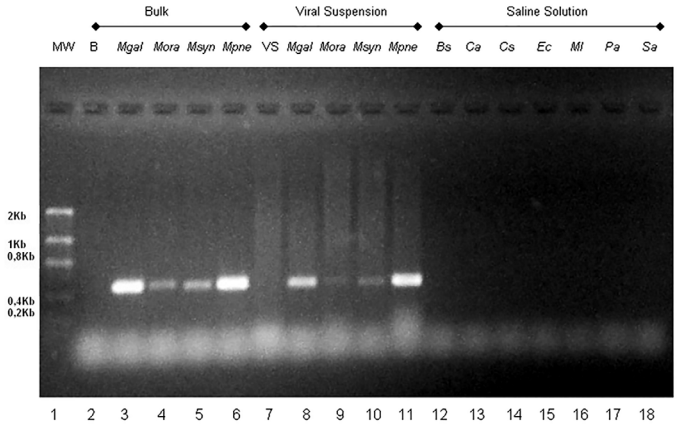

Figure 3. Electrophoretic analysis of PCR products of one of the two replicates from the selectivity assay. Whole volume was applied on 1 % agarose gel. Lane 1. "Low Mass" molecular weight marker. Lane 2. Bulk negative control. Lane 3-6. Bulk spiked with 25 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*, respectively. Lane 7. Viral suspension negative control. Lane 8-11. Viral suspension spiked with 25 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumonia*, respectively. Lane 12-18. Saline spiked with 200 CFU of *Bacillus subtilis*, *Candida albicans*, *Clostridium sporogenes*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively

Limit of detection (LOD)

Aiming at establishing the minimal mycoplasma concentration detectable through the proposed methodology, we used the intermediary products which were intentionally contaminated with decreasing concentrations of each of the mycoplasma species. This assay was prepared in duplicate.

For *M. gallisepticum* and *M. pneumoniae* the determined LOD in both bulk and viral suspension samples was roughly 3.1 CFU; and regarding *M. orale* and *M. synoviae*, this limit value was roughly 3.1 CFU for bulk and 12.5 CFU for viral suspension. LOD was not specifically defined when concentration of 3.1 CFU generated PCR products, suggesting an even lower limit of detection.

Sensitivity, specificity, positive predictive value, negative predictive value, analytical accuracy and Kappa index

In order to evaluate the method variability according to sensitivity, specificity, positive predictive value, negative predictive value, analytical accuracy and Kappa index, this electrophoretic procedure was repeated 14 times using different samples, i. e., various seed lot vials dilution in the vaccine intermediates at distinct days, and the test running by distinct analysts. In none of these analyses, false-results were observed, as exemplified in **Figure 4**.

Of 140 assayed samples, 112 showed true-positive profile and 28 exhibited true negative status, i.e. all of the analyzed samples evidenced true status. Therefore, the parameters of sensitivity, specificity, positive predictive value, negative predictive value and analytical accuracy demonstrated 100 % performance and Kappa index=1.

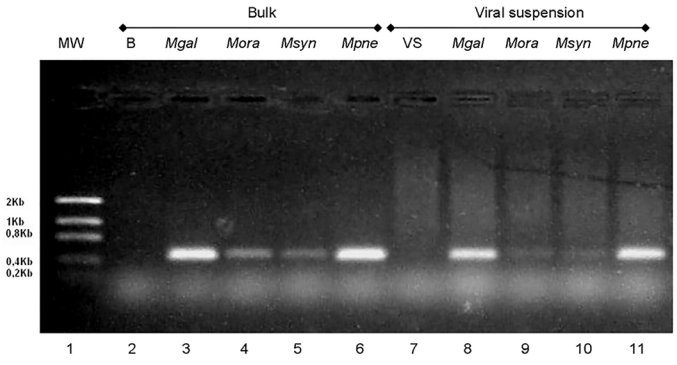

Figure 4. Electrophoretic analysis of PCR products in one of 14 replicates for assessing the sensitivity, specificity, positive predictive value, negative predictive value, analytical accuracy and Kappa index. Whole volume was applied on 1 % agarose gel. Lane 1. "Low Mass" molecular weight marker. 2. Bulk negative control. 3-6. Bulk spiked with 30 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*, respectively. 7. Virus suspension- negative control. 8-11. Viral suspension spiked with 30 CFU of *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*, respectively

Quality Control (QC) and Assurance

The standardized operational procedure (SOP) of the whole mycoplasma detection methodology was developed. This protocol has been registered at the Quality Assurance Department of Bio-Manguinhos/Fiocruz, Rio de Janeiro-RJ, and it has being followed throughout the validation experiments. A validation report for documenting the assays has been prepared, registered and then this methodology has being

incorporated into the QC assays performed in the YF vaccine produced in Bio-Manguinhos/ Fiocruz, since its validation in May 2008. And from then, the PCR approach has been used together with the mycoplasma culture classical method, and both show compatible results.

DISCUSSION

Biological and medical products differ from chemical drugs which usually cannot be characterized molecularly; starting methods such as bacteria, viruses, or genetically modified microorganisms are of enormous complexity, besides they are capable to vary according to the preparation methodologies used for. The certain kind of these products, such as the vaccines against transmissible disease, are also administered to healthy individuals, often in children at the beginning of their lives**.** Thus, a strong emphasis must be placed on their quality to ensure, to the greatest extent possible, that they are efficacious in preventing or treating life-threatening disease, without themselves causing harm. The WHO has a whole set of requirements for QC of the vaccines produced throughout the world, including the YF vaccine[5]. Among the QC assays required for YF vaccine, the detection of mycoplasma is mandatory**.** According to the WHO requirements the current widely applied mycoplasma detecting method is the microbiologic culture, which takes about 35 days to provide results[4]. The WHO Technical Report Series number 872 was under evaluation to investigate what needs to be changed[16].

The PCR method has been routinely employed for detecting microorganism in disease diagnosis, food and environment assessment, being considered as reliable technique for these purposes. The use of PCR amplification has revolutionized the ability to trace contaminants. However, considering that these techniques detect not only the viable organisms, great care has to be exercised when analyzing the results and their impact on the products safety. Mycoplasma detection in cell cultures has long been the subject of research[7,8,12-15,17-21]. This report is the first one to describe a molecular approach for detecting mycoplasma in YF vaccine. Concerning the bacterial culture procedure for mycoplasma detection, the WHO requirements for the medium approval establishes the ranges from 20 to 40 CFU for liquid culture medium and from 200 to 400 CFU for solid culture medium[5]. In the present study we have taken into account these approval ranges for PCR validation.

The purpose of this study was to improve the methodology for detecting mycoplasma, thus streamlining the QC assays used during the YF vaccine production. And for accomplishing this end**,** we selected four different techniques of PCR assays previously described[12-15]. We evaluated the ability of each protocol to detect mycoplasma DNA in spiked YF vaccine intermediates, aiming at developing a standardised protocol as part of the routine QC. None of these methods, even performed precisely as described by the authors, it will be unable to meet the objectives at the first evaluation. It is plausible to assume that the first negative results should occur due to the presence of inhibitors in DNA samples.

PCR inhibition is a common drawback for implementing the PCR-based techniques[22,23]. As the YF vaccine is produced in chicken embryos, the previously reported PCR inhibitors present in blood, flesh and skin[24,25] could interfere with DNA amplification. Inhibitor compounds as heme composites, alkaline haematin, degraded serum proteins, haemoglobin, urea, fats and calcium have been previously found to remain in DNA samples regardless of the method used for nucleic acid purification[26,27]. Iron-containing inhibitors (e.g. iron chloride, lactoferrin, haemoglobin and haemin) compete with the magnesium in the PCR reaction, thus an increase in magnesium chloride concentration is required to make the reaction to proceed[28]. Additionally, polysaccharide complex and immunoglobulin contaminants can bind to the DNA, impairing the effectiveness of the PCR annealing step**,** and hence demanding changes in the cycling temperatures[27]. In addition to the activity of inhibitors, the PCR assay itself is highly sensitive to minor changes in the protocol, meaning that

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subtle variations may produce higher amounts of amplicon or even no amplicon at all[23].

Based on these negative results, the protocols were modified, attempting to get a positive amplification. The modification was successful only for DSMZ primers set, which were optimized by testing the different magnesium and primer concentrations and the cycling temperatures. In order to avoid the variability, all of the modifications were carried out using the same extracted DNA as template. The parameters validation and their acceptance criteria were based on the United States Pharmacopoeia[10] and scientific literature[29,30]. In the present study we validated an alternative method providing a faster outcome and highly sensitive test for detecting mycoplasma in YF vaccine QC, according to the selectivity, LOD, sensitivity, specificity, positive and negative predictive values, analytical accuracy and Kappa Index. Robustness of the PCR assay was not assessed in a formal validation, but it was retrospectively evaluated after the method has been developed.

The selectivity measures the answer degree of the assay regarding the target and non-target analytes. This parameter met the expected profile of this study, exhibiting DNA amplification when the genomic DNAs from the four mycoplasma species were used, parallel to the no-amplicon resulting from the negative YF vaccine intermediate controls amplification. The mycoplasma DNA concentration was derived from 25 CFU of each mycoplasma previously to the DNA isolation; and this is similar to that required by WHO for demonstrating the nutrient properties of the liquid culture media for mycoplasma detection[5]. Additionally, unrelated bacteria and fungi species spiked into YF vaccine intermediates yielded no DNA amplification. This test assured the ability of our standardized PCR protocol in producing amplicons generated from template DNA of YF vaccine spiked with *Mycoplasma spp*.**.** Besides, the unspecific amplification was not observed by using either YF vaccine without mycoplasma contamination or with contaminating agents as bacteria and fungi, e.g., *C. sporogenes*, *M. luteus, B. subtilis*, *S. aureus*, *C. albicans*, *E. coli* and *P. aeruginosa*. These microorganisms were chosen for evaluating the selectivity test, based on the controls of the growth promotion assay for approving the culture media to perform the sterility tests[31].

Our study also allowed an appropriate LOD for further supporting this new alternative method. LOD was determined in spiked vaccine intermediates, including four mycoplasma species, i.e., *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*. The *M. gallisepticum* and *M. pneumoniae* spiked samples provided the amplification of 16S ribosome gene in a bacterial concentration as low as 3.1 CFU in both vaccine intermediates. From the both species the detected concentration was even below 3.1 CFU, with a worth noting high intensity of bands acquired from the amplification in the spiked YF vaccine bulk, when compared to those finding from the spiked YF-vaccine viral suspension amplification. Such difference might be occurred due to the existence of PCR inhibitors in the YF vaccine suspension extract, implying that the detection in viral suspension could be further optimized. Regarding the YF-vaccine intermediates matrices, the bulk is approximately one decimal dilution of viral suspension with stabilizing solutions. Thus, it is reasonable to assume that the distinct efficiencies in mycoplasma amplification might be related to the amount of inhibitors in each of these two analytes, as discussed above. In contrast, *M. orale* and *M. synoviae* showed amplicons with equally lower threshold of detection, i.e., 3.1 and 12.5 CFU for spiked YF vaccine bulk and viral suspension, respectively. These data suggest the strong avidity of the primer set described by Uphoff and Drexler[13] for *M. gallisepticum* and *M. pneumoniae* as compared to *M. orale* and *M. synoviae*.

Our results regarding validation parameters were analysed by means of 2x2 matrix based on Chi-Square method, i.e., the contingency table, defining the sensitivity, specificity, positive predictive value and negative predictive value, analytical accuracy and Kappa index[29,30]. These contingency statistics confer reliability to the validation study of qualitative methods such as end-point PCR, providing a quantitative measurement for the magnitude of agreement

among the evaluated samples. The number of repetitions of this procedure directly influences in its representativeness. All of the aspects regarding the contingency table yielded values of 100 %, and no false negative or false positive results were found. Contingency tables with a wide range of agreement rates were described in previous studies on PCR validation[29,32].

In the present study, the stocks of live bacteria (seed lots) were used for quantifying mycoplasma by means of plating and serial dilutions counting before and after samples freezing. The quantification of the seed lots took into account only the plates ranging from 50 to 300 colonies per plate, and it was performed for analyzing the reproducibility[33]. These seed lots were used as a contamination source for method validation, in both the positive controls and the spiked samples. Thus, the tests were performed using the intermediary products spiked with previously determined quantities of live bacteria. Therefore, the reproducibility of the performed tests was also improved by seed lots approach, i.e. for the each test setting one vial was thawed and diluted into the respective intermediary product before the DNA isolation. All of the tests were carried out employing pure samples (without mycoplasma) as negative controls.

Additionally, the mycoplasma culture media employed at QC Department of Bio-Manguinhos was suitable to promote the growth of their four species. According to the ATCC[34], three different media should be applied to this end. The European Pharmacopoiea also describes more than one media to performing the mycoplasma control[35]. However, the previous study showed that no difference was found in the mycoplasma growth in different culture media[36]. Therefore, in addition to the PCR protocol, we suggest to use a simple and straightforward method for quantifying mycoplasma using a single culture media that provides the growth of four tested mycoplasma species *M. gallisepticum*, *M. orale*, *M. synoviae* and *M. pneumoniae*.

The mycoplasma quantification by culture methods remains as a complex matter of debate; and among the PCR approaches employed for alternative QC, the genome copies has been chosen as a means for bacterial counting[37,38]. Even so, there is a ratio between the genome copies versus the CFU**,** which is at most minimized only. Degraded genomic DNA is critical for a reliable correlation of the genome copy numbers, and the statistical approaches should be applied to accept these results. Many biases have to be evaluated when genome copies are employed, as biological features of mycoplasma strain, phase of mycoplasma growth, growth conditions and mycoplasma sample handling after the cultivation[8]. Besides, the copy number of the 16S rRNA gene differs among the different species of mycoplasma, introducing as such a further bias to the genome copy number evaluation[39,40]. Additionally, the yield provided by the different DNA isolation methods may vary widely, generating outputs that do not correlate to the real bacterial amounts in the samples**,** if the DNA isolation method is changed. As such, our study aimed at performing an evaluation based on WHO requirements referring to the CFU counting in order to minimize biases.

The use of both methods together for performing the bacteriological and molecular analyses and aiming at the same purpose, this investigation should be an advantageous alternative**.** Thus, the present study provided an even more accurate ranking of certainty and safety characteristics regarding the absence of mycoplasma in YF vaccine. Moreover, the viability is not considered in PCR assays, whilst the culture methods provide mycoplasma detection solely from the viable bacterial cells. There are perspectives to assess the real time PCR methodologies in comparison to the validated assay.

Since 2008, when this molecular method based on DSMZ primers has been validated, over than 2,000 YF vaccine intermediate samples were analyzed at the Bio-Manguinhos/ Fiocruz - QC Department, by means of the parallel with both PCR and mycoplasma culture methods.

CONCLUSION

This paper is the first successful demonstration of the use of PCR-based QC assay for evaluating the YF vaccine produced

Publicação contínua *on line*: número de páginas sem efeito para citação
On line continuous publishing: page number not for citation purposes

in embryonated eggs. This validation process is clearly not a final step on the mycoplasma QC, as we aim at constantly improving the techniques to contribute for increasing the confidence assurance of the YF vaccine produced in Bio-Manguinhos, as well as to establish the best QC standards of vaccines and health products in general.

ACKNOWLEDGEMENTS

This work was supported by Bio-Manguinhos-Fundação Oswaldo Cruz/ Ministério da Saúde/ Brasil. We thank Dr. Ricardo Galler who provided insight and expertise that greatly assisted the research and for his comments that improved the manuscript.

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