Acta Scientiae Veterinariae, 2016. 44: 1343.

RESEARCH ARTICLE
Pub. 1343

ISSN 1679-9216

An Assessment of Microbiological Methods to Test Sterility of Foot-and-mouth Disease Vaccines Produced in Brazil

Carla Rosane Rodenbusch, Luiz Roberto da Silveira, Álvaro Ricardo Bavaresco & Marcus Vinícius Burgel Sfoggia

ABSTRACT

***Background*:** Foot-and-mouth vaccines are an important tool in the control and eradication of the disease. In order to be commercialized, vaccines produced in Brazil undergo an evaluation process by health authorities, which includes sterility testing, residual active virus, potency, thermal stability, volume and non-structural protein activity. Sterility tests described in the Brazilian Pharmacopeia and by the World Organization for Animal Health (OIE) include direct inoculation and membrane filtration methods. The objective of the present study was to evaluate these two methods used to analyze sterility of vaccines against foot-and-mouth disease produced in Brazil.

***Materials, Methods & Results*:** Vaccines produced by the six main laboratories in Brazil were initially tested for filtration capacity. The sensitivity of the two techniques was determined artificially contaminating vaccines using known bacterial concentrations. Vaccines (9 bottles) from the same manufacturer were inoculated with 5 mL of steady-state growths of *Pseudomonas aeruginosa*, *Candida albicans*, and *Clostridium sporogenes* to final concentrations of 0.1, 1 and 10 CFU/mL and a final volume of 55 mL. Bottles were manually shaken for 1 min to complete homogenization of contents. Then, 10 mL of each flask were used in assessment of the direct inoculation method, and 10 mL were used to evaluate the membrane filtration technique. Direct inoculation was carried out inoculating 1 mL of the experimentally contaminated vaccine in five tryptic soy broth (TSB) and fluid thioglycollate medium (FTM) bottles. The membrane filtration technique was carried out filtrating 10 mL of the challenged vaccines in a peristaltic pump system (Steritest™ Pump System), where vaccines were initially solubilized in Triton X-100 to promote filtration. Next, membranes are incubated in TSB and FTM. These use two types of culture medium, tryptic soy broth (TSB) and fluid thioglycollate medium (FTM), with incubation times of 20-25ºC and 30-35ºC, respectively, to detect fungi, yeasts, and aerobic and anaerobic bacteria. The medium is incubated for 14 days, to enable the detection of slow-growth microorganisms that may be in a latent stage or weakened due to the extreme conditions of the production process (like the use of cleaning and disinfection agents, ultraviolet light, and preservers, for instance). All vaccines were effectively filtered in the Steritest™ Pump System. Membrane filtration and direct inoculation presented the same sensitivity to detect yeasts (0.1 CFU/mL) and anaerobic organisms (1 CFU/mL). For the detection of aerobic organisms, membrane filtration was 100 times more sensitive, compared to direct inoculation.

***Discussion*:** The specialized literature also reports that, apart from the higher sensitivity, membrane filtration affords to reduce contamination during the procedures, since it is carried out in a closed system. In addition, it is indicated in the analysis of large sample volumes. Moreover, membrane filtration reduces the occurrence of false positive results, since it removes the excess vaccine volume from the culture medium, which may be mistaken for turbidity caused by bacterial growth. In this sense, the membrane filtration technique is more appropriate in the control of vaccine sterility in foot-and-mouth disease prevention strategies, and is an interesting tool to improve quality control of the product.

Keywords: Foot-and-mouth disease, vaccine, direct inoculation, membrane filtration, sterility.

Received: 4 August 2015 Accepted: 29 December 2015 Published: 22 January 2016

Laboratory of Foot-and-mouth Disease Vaccines, National Livestock and Agriculture Laboratory, Ministry of Agriculture, Livestock and Food Supply, Brazil. CORRESPONDENCE: C.R. Rodenbusch [carlarodenbusch@yahoo.com.br - Tel.: +55 (51) 3779-7838]. Laboratory of Foot-and-mouth Disease Vaccines, National Livestock and Agriculture Laboratory Ministry of Agriculture, Livestock and Food Supply, Rio Grande do Sul, Brazil. Estrada Retiro da Ponta Grossa, n. 3036. CEP 91780-580 Porto Alegre, RS, Brazil.

INTRODUCTION

Foot-and-mouth disease is one of the most important conditions that affect split-hoofed animals. It infects cattle, buffalo, pigs, sheep, and goats, as well as approximately 70 wild species [7]. This vesicular disease is caused by a virus of the genus *Aphtovirus*, family *Picornaviridae*, with high infection rates in livestock worldwide [8]. Vaccines against foot-and-mouth disease are an important tool in the control of the condition in endemic regions [14]. Currently, the vaccine is produced as a primary two-phase oil-in-water emulsion. The aqueous phase contains virus inactivated with a first-order inactivator, while the oil phase is prepared with mineral oil as adjuvant [1]. In Brazil, vaccines can only be commercialized after official evaluation procedures by the Ministry of Agriculture, Livestock and Food Supply. These include sterility, volume, residual active virus, potency, thermal stability and non-structural protein activity [2]. Sterility testing described in the Brazilian Pharmacopeia [3] and the World Organization for Animal Health [9] include direct inoculation and membrane filtration methods. These use two types of culture medium, tryptic soy broth (TSB) and fluid thioglycollate medium (FTM), with incubation temperature of 20-25ºC and 30-35ºC, respectively, to detect fungi, yeasts, besides aerobic and anaerobic bacteria [3,9].

The aim of the present study was to evaluate the efficiency of two microbiological methods (direct inoculation and membrane filtering) described in the literature in the quality control of sterility of vaccines against foot-and-mouth disease produced in Brazil.

MATERIALS AND METHODS

Vaccines

All samples used had been tested using the inoculation method by manufacturers as part of their quality control routine, with negative results. However, filterability was of foot-and-mouth disease vaccines (commercialized in 50 mL bottles) from six different manufacturers was assessed. The vaccines tested were from the main Brazilian producers and exporters of the medicament.

Strains and preparation of microorganism dilutions

Yeasts as well as Gram-positive, Gram-negative, aerobic, anaerobic and spore-former microorganisms were used. All were from the American Type Culture Collection (ATCC) (Table 1). Serial decimal dilutions of steady-state cultures were prepared using peptone saline 0.1% to obtain 10, 1 and 0.1 colony forming units (CFU)/mL. These solutions were used to evaluate effectiveness of growth media and to experimentally challenge vaccines so as to assess sensitivity of the methods direct inoculation and membrane filtering. Colony forming units were counted in tryptose sulfite cycloserine agar (TSC)[1] for *Clostridium sporogenes* and plate count agar (PCA)[2] for the other microorganisms.

Growth media and reactants

The TSB, FTM, and fluid D media, as well as the cassettes used in the membrane filtration technique were purchased from Millipore, USA. Triton solutions (Triton X-100®)[3] 0.1% and 10% were prepared to test the membrane filtration technique. Microorganisms were counted using the media peptone water and TSC and PCA.

Effectiveness of growth media

To assess whether growth media were able to promote microbial development, 1 mL of the 0.1, 1, and 10 CFU/mL solutions of the microorganisms *P. aeruginosa* and *C. albicans* was inoculated in TSB and FTM, while *C. sporogenes* was inoculated in FTM for 14 days, at temperatures given in Table 1. The same solutions were plated on PCA and TSC (anaerobiosis) and incubated at 30-35ºC for 48 h. Then, colonies were counted and concentrations were evaluated.

Negative controls were used in all analyses. Growth media were incubated as described, but without inocula.

Experimental challenge of vaccines

Vaccines (9 bottles) from the same manufacturer were inoculated with 5 mL of steady-state growths of *P. aeruginosa*, *C. albicans*, and *C. sporogenes* to final concentrations of 0.1, 1 and 10 CFU/mL and a final volume of 55 mL. Bottles were manually shaken for 1 min to complete homogenization of contents. Then, 10 mL of each flask were used in assessment of the direct inoculation method, and 10 mL were used to evaluate the membrane filtration technique.

Direct inoculation method

To determine sensitivity, bottles containing 20 mL of TSB[2] and of FTM[2] were inoculated (in five replicates) with 1 mL of the vaccines experimentally challenged with *P. aeruginosa*, *C. albicans*, and *C. sporogenes*. Tubes were incubated at the tempera-

tures given in Table 1, for 14 days, and readings were made every 2 days. Microbial growth was observed as turbidity of the TSB medium and the turbidity or fading of the pink halo in the FTM medium.

Membrane filtration method

Filterability of vaccines was evaluated using the Steritest™ method. Vaccines (50 mL) of six different manufacturers were submitted to the same filtration protocol. All analyses were carried out in the compact Steritest™ pump system[4]. Triton[3] 10% (50 mL) was used to moist the membrane in each canister. Then, 10 mL of the vaccine were homogenized with 50 mL of Triton 10%. After filtration, canisters were washed with 100 mL of Triton 10% twice and 100 mL Triton 0.1% once. Finally, canisters were washed with 100 mL fluid D, to remove excess Triton, and incubated with 100 mL of FTM and TSB for 14 days, respectively, at 30-35ºC and 20-25ºC.

Sensitivity was analyzed submitting the vaccines experimentally challenged with *P. aeruginosa*, *C. albicans*, and *C. sporogenes* to the protocol described above.

Readings were carried out every two days. Microbial growth was observed as turbidity of the TSB medium and the turbidity or fading of the pink halo in the FTM medium.

Confirmation of contamination

Confirmation of turbidity of the culture media used in the comparison between direct inoculation and membrane filtration and caused by growth of microorganisms inoculated in the vaccine was confirmed seeding the FTM medium in TSC agar (under anaerobic conditions) and incubation for 48 h at 30-35ºC. In turn, the TSB medium was seeded on PCA agar and potato agar plates, with incubation at 30-35ºC (48 h) and at 20-25ºC (72 h), respectively.

Table 1. List of microorganisms used in the evaluation of direct inoculation and membrane filtration.

	Strain ID	Anaerobic/aerobic	Gram stain	Incubation temperature (°C)
Pseudomonas aeruginosa	ATCC27853	aerobic	Gram-negative rods	30-35
Clostridium sporogenes	ATCC11437	anaerobic	Gram-positive rods	30-35
Candida albicans	ATCC10231	aerobic	Yeast	20-25

Table 2. Results (CFU/mL) of sensitivity assays for direct inoculation and membrane filtration.

		C. albicans			*P. aeruginosa*			*C. sporogenes*		
		10	1	0.1	10	1	0.1	10	1	0.1
Direct inoculation	FTM	5/5	5/5	5/5	5/5	0/5	0/5	5/5	5/5	0/5
	TSB	5/5	5/5	5/5	5/5	0/5	0/5	NA	NA	NA
Membrane filtration	FTM	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1
	TSB	1/1	1/1	1/1	1/1	1/1	1/1	NA	NA	NA

NA: not applicable.

RESULTS

The TSB and FTM media promoted the growth of *P. aeruginosa*, *C. albicans*, and *C. sporogenes*, with the three solutions used (0.1, 1, and 10 CFU/mL).

Independently of manufacturer, all vaccines were filterable in the Steritest™ system, and no negative control presented bacterial growth.

To determine the sensitivity of direct inoculation and of membrane filtration, the two techniques were conducted in parallel, using the same vaccine samples experimentally challenged with bacterial contamination. Table 2 shows how the two methods compare, in terms of sensitivity. The different microbial dilutions were tested (quintuplicates) in direct inoculation. Replicates of the same dilution presented the same result (either positive, or negative).

In order to confirm that turbidity of media was caused by microorganisms inoculated in vaccines, cultures were seeded on solid medium. Bacterial growth in dishes containing agar TSC were typical of *Clostridium* sp. (black colonies), while the potato agar dishes exhibited common yeast cultures (smooth,

shiny, white-to-yellowish cultures). PCA that exhibited bacterial growth were used in Gram staining and biochemical assays. *Candida albicans* colonies on PCA had typical morphology and color in the Gram staining (pleomorphic and Gram-positive staining), while colonies of *Pseudomonas aeruginosa* were rod-shaped, Gram-negative, and positive in the oxidase assay.

DISCUSSION

Like any other biologically sourced product, vaccines against foot-and-mouth disease are produced aseptically and tested for sterility during the various production stages, since they cannot be submitted to one single final sterilization process. Therefore, sterility evaluations, together with asepsis in processes, environmental surveillance, and the use of sterilized equipment and reactants ensure that the end product meets sterility requirements [10]. Sterility implies the absence of any viable microorganism able to pose hazards during or after administration or use of a product [12]. A 10^{-6} sterility level is usually acceptable in processes adopted by the pharmaceutical industry. In other words, this means less than one viable microorganism in one million units produced [5]. We evaluated solutions of 10, 1, and 0.1 CFU/mL, as recommended in literature [10]. Sensitivity of the membrane filtration technique was identical to that of direct inoculation in the detection of yeasts (0.1 CFU/mL) and of anaerobic microorganisms (1 CFU/mL). As for the detection of aerobic microorganisms, membrane filtration was 100 times more sensitive than direct inoculation. Similarly, the first technique is more sensitive when compared with plating on solid medium [6] and with direct inoculation [3,4]. In fact, membrane filtration detects as many as 9% more positive samples (at 1 CFU/mL), compared to direct inoculation [10].

The inactivator used in the production of the vaccine against the foot-and-mouth disease virus also acts against other microorganisms, reducing the microbial load of experimentally challenged vaccines [3]. Moreover, the number of viable microorganisms drops with time, due to the kinetic of bacterial growth [13]. With a view to simulating contamination during the production process, we chose to inoculate vaccines with known concentrations of microorganisms. Besides, vaccines were analyzed immediately after experimental challenge with these pathogens, in order to minimize the loss of viability of these microorganisms. For this reason, the higher sensitivity of the membrane filtration technique is explained by the fact that the microorganism is kept from the product during incubation (which does not occur in direct inoculation). Ultimately, this segregation allows weakened pathogens to recover more efficiently [10].

Membrane filtration has some advantages over direct inoculation: (i) it reduces contamination during analysis, since it is carried out in a closed peristaltic pump [3]; (ii) it enables the analysis of larger samples [11]; (iii) it reduces the chances of false positives, since excess vaccine is removed from the medium, which otherwise would increase turbidity, influencing results [3].

CONCLUSION

In conclusion, the sensitivity of the membrane filtration technique is similar to that of direct inoculation in the detection of fungi, yeasts, and anaerobic bacteria in vaccines against foot-and-mouth disease, though it is more sensitive to detect aerobic microorganisms. In this sense, the membrane filtration technique is the most appropriate in the determination of sterility in vaccines against foot-and-mouth disease.

MANUFACTURERS

[1]Oxoid. Basingstoke. Hampshire, UK.

[2]Himedia. Curitiba, PR, Brazil.

[3]Sigma-Aldrich. Sant Louis, MO, EUA.

[4]Merck Millipore. Darmstadt, Alemanha.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1 **Abaracón B. & Olascoaga R.C. 1984.** Vacinas contra a febre aftosa. *A Hora Veterinária*. 17: 45-52.

2 **Ministério da Agricultura Pecuária e Abastecimento. 2008.** Instrução Normativa 50/2008 de 24/09/2008. Regulamento Técnico para a Produção, Controle da Qualidade, Comercialização e Emprego de Vacinas Contra a Febre Aftosa, 7p. Disponível em: <https://www.diariodasleis.com.br/busca/exibelink.php?numlink=1-77-23-2008-09-23-50>. [Acessed online June 2015.]

3 **Agência Nacional de Vigilância Sanitária. 2010.** Farmacopéia Brasileira. 5.ed. v.1. *Métodos biológicos, Ensaios biológicos e Microbiológicos*. pp.256-260. Disponível em: <http://www.anvisa.gov.br/hotsite/cd_farmacopeia/pdf/volume1.pdf>. [Acessed online May 2015].

4 Cortés A., Sandino C. & Arias J. 2003. Validación de la prueba de esterilidad para vacunas virales preparadas em vehiculos oleoso y acuoso. *Revista de la Faculdad de Farmacia*. 45: 36-43.

5 European Commission. 2012. *Guide to Good Manufacturing Practice for Medical Products for Human and Veterinary Use. Manufacture of Sterile Medicinal Products*. 16p. Disponível em: <http://ec.europa.eu/health/files/eudralex/vol-4/2008_11_25_gmp-an1_en.pdf >. [Acessed online May 2015].

6 Hernandez-Lopez J., Guzman-Murillo A. & Vargas-Albores F. 1995. Quantification of pathogenic marine vibrio using membrane filter technique. *Journal of Microbiology Methods*. 21: 143-149.

7 Jamal S.M. & Belsham G.J. 2013. Foot-and-mouth disease: past, present and future. *Veterinary Research*. 44: 116-129.

8 MacLachlan N.J. & Dubovi E.J. 2011. Picornaviridae. In: *Fenner's Veterinary Virology*. London: Academic Press, pp.431-435.

9 OIE - World Organization for Animal Health. 2013. Manual of diagnostic test and vaccines for terrestrial animals. Tests for Sterility and Freedom from Contamination of Biological Materials, 10p. Disponível em: <http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.07_TESTS_FOR_STERILITY.pdf>. [Acessed online June 2015].

10 Parveen S., Kaur S., David S.A.W., Kenney J.L., McCormick W.M. & Gupta R.K. 2011. Evaluation of growth based rapid microbiological methods for sterility testing of vaccines and other biological products. *Vaccine*. 9: 8012-8023.

11 Pinto T.J.A., Kaneko T.M. & Ohara M.T. 2003. *Controle biológico de qualidade de produtos farmacêuticos, correlatos e cosmético*. 2.ed. São Paulo: Atheneu, 325p.

12 Sharp J. 1995. What do we mean by sterility? *PDA Journal of Pharmaceutical Science and Technology*. 49: 90-92.

13 Von Woedtke T. & Kramer A. 2008. The limits of sterility assurance. *GMS Krankenhaushygiene*. *Interdisziplinär*. 3: 1-10.

14 Willems T., Lefebvre D.J., Goris N., Diev V.I., Kremenchugskaya G.P., Haas B. & De Clercq K. 2012. Characteristics of serology-based vaccine potency models for foot-and-mouth disease virus. *Vaccine*. 30: 5849-5855.