# MEASUREMENT OF ANTIBIOTIC LEVELS IN FOOT-AND-MOUTH DISEASE OIL VACCINES BY CHEMICAL METHODS

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***SUMMARY.*** **Quality control of foot-and-mouth disease (FMD) vaccines currently comprises sterility, immunogenicity and innocuity tests but no assessments of potentially allergenic components. This paper reports the results of a study to select the optimal working conditions for rendering thin layer chromatography (TLC) useful for determining the levels of penicillin, neomycin and polymyxin in FMD oil vaccines. Procedures are described for breaking vaccine emulsions and for extracting, purifying, concentrating, identifying and quantifying the antibiotics contained in them. Subsequently, commercially available FMD oil vaccines were examined by these procedures. Findings are discussed in terms of the application of TLC for quality control of immunogens and for studying post-vaccinal reactions.**

In general, vaccine control laboratories basically analyze three aspects of the immunogens manufactured within their sphere of influence. Thus, foot-and-mouth disease (FMD) vaccine *potency* tests, known as podal generalization tests (*5,20*) require that vaccines are able to protect 75 percent of bovines following viral challenge at 90 days after primary immunization. To lower costs and prevent environmental dissemination of virus, increasing use is being made currently of the expected percent protection based on mouse protection tests (*9*) for this purpose.

Quality control of FMD vaccines also includes *sterility* tests based on the inoculation of culture media to detect microbial contaminants that may affect vaccine immunogenicity and stability, or alternatively, infect the animals receiving them. Finally, *innocuity* tests are carried out to prevent the great risks which vaccines containing infective viral particles represent, and to analyze the tolerance of animals to vaccination and revaccination (*1,2,5,10*).

Since the administration of FMD vaccines on a regular periodic basis was initiated, a diversity of post-vaccinal problems which include local or generalized manifestations compatible with allergic reactions, have been recorded (*3,4,6-8,11,13-16,19*). Although their frequency seems to be of minor significance, they may present a problem in some circumscribed areas. Nevertheless, we are

Reprint requests to:
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some circumscribed areas. Nevertheless, we are not aware that the possibility has been contemplated of incorporating tests to determine the levels of potentially allergenic components in these and other vaccines. This would be of interest to establish associations between the values of different allergens and the appearance and frequency of reactions; to define the levels of these substances which are compatible with the absence or reduction of reactions; to improve vaccine quality control procedures, and consequently, favor the progress of anti-FMD immunization programs.

Studies reported herein were carried out to develop a procedure which would permit the determination of antibiotic levels in different lots of FMD vaccines. Priority was assigned to these products since among vaccine components, they have been most frequently incriminated ( *3,4,6,8, 11,13-15,19*) with the appearance of post-vaccinal allergic manifestations in livestock.

## MATERIALS AND METHODS

Considering its widely recognized usefulness for identifying and classifying antibiotics, separating them from mixtures and controling their purity, thin layer chromatography (TLC) was selected for determining the presence of antibiotics in FMD vaccines, employing chemical methods to identify the zones which correspond to the different antibiotics. Furthermore, selection of TLC was based on the following practical considerations: its simplicity and ease of performance; its capacity to cover a wide range of antibiotics, its sensitivity, which allows for detection of small amounts; it may be performed with equipment usually available in microbiology laboratories; it does not require rare, dangerous, or costly reagents, nor expensive equipments, and it has an adequate repeatibility, reproducibility and specificity (*17,18*).

Initially, the working approach consisted of selecting the optimal working conditions for each of the following four stages of the process: breaking the emulsion, extraction of the antibiotics, their purification and concentration, and finally, their identification and quantification by TLC.

After defining these conditions, the procedure was applied to determine the antibiotic levels present in a random selection of FMD vaccine lots submitted by producers to the official control agencies of Argentina and Colombia (to whom we are grateful for kindly providing the samples used for this purpose).

For use in these four stages, standards were prepared with the three antibiotics (*12*) most frequently incorporated into FMD vaccines available commercially in South America, specifically the following: (a) potassium G penicillin standard solution at 1.00-0.50 and 0.25 mg/ml; (b) standard neomycin sulphate standard solution at 2.00-1.00 and 0.5 mg/ml; (c) polymyxin B standard solution at 0.100-0.050 and 0.025 mg/ml.

Furthermore, an FMD oil vaccine manufactured at the Vaccine Pilot Plant of the Pan American Foot-and-Mouth Disease Center (PANAFTOSA) was also used as a reference reagent to set up the four stages of the procedure. During the preparation of this and the other PANAFTOSA vaccines used in this study, 250 IU of penicillin, 0.56 mg of neomycin sulfate, 125 IU of polymyxin sulfate, and 6.2 ng of fungizone were added per every 5 ml of vaccine (which consisted of equal parts of the inactivated virus suspension in tissue culture medium, and the oil phase).

Priority was assigned to the design and optimization of the general procedures for *extraction* of antibiotics from the FMD oil vaccine used as reference. To this effect, procedures were tested for liquid phase extraction at different pH with different buffer systems. Similarly, several systems for *purification* of the aqueous extract of vaccines by partition with organic solvents and by development in opposite directions (anti-parallel development) in TLC and by multiple development in the same phase.

The possibility was first contemplated of using an aqueous phase extraction system and subsequent purification using multiple development by TLC. Nevertheless, recoveries and the complete separation of phases were not at all satisfactory, since the effective destruction of the emulsion was not achieved with chloroform. The

persistence of a very stable intermediate phase of variable dimension became evident, placing the limit of detectability or the system. This required incorporating internal standardization, rendering it cumbersome and thus impractical, and conveyed the need to search for a substance adequate for this purpose. Accordingly, these difficulties were obviated by breaking the emulsion with dichloromethane, and purifying by solid phase extraction (SPE).

On the other hand, extracts were concentrated for the purpose of reaching levels adequate for the selected visualization method. To this effect, the reduction in volume by *concentration* was assayed using air currents at low temperatures or azeotropic distillation (water/ethanol and water/acetone) also at low temperatures in order not to alter the structure of the antibiotics. On this basis, it was found that concentrating the aqueous phase by air currents at low temperatures was more effective for reducing the volume of the vaccine extract.

For the purpose of optimizing the method of separating antibiotics which were of interest by chromatography, a variety of systems for development over a *normal phase* were assessed (seven mixtures of binary, ternary and quarternary solvents) as well as another on *reverse phase*. The conclusion was reached that the system in normal phase using silicagel 60 plates was the most adequate. Options made consisted of using a quaternary mobile phase for neomycin and polymyxin, and a binary system for penicillin and its related antibiotics. Because of the characteristics of these two antibiotic groups, it was necessary to use different chromatographic development systems and staining reactions.

To visualize the localization of antibiotics in the TLC plates and to permit detection levels of approximately 2 μg of each antibiotic, two methods for developing them were assayed. These techniques were based on the formation of *colored compounds* (using sodium azide/$I_2$, $I_2$/starch, or ninhydrin-acetic acid) and in the formation of *fluorescent derivatives* (by derivation with fluorescamine) to improve the sensitivity of detection.

On this basis, developing with $I_2$/starch was selected for antibiotics of the penicillin family, and ninhydrin/acetic acid for the polymyxin and neomycin families. Also, it was concluded that the correct functioning of the system with fluorescence justifies its usefulness as a complement or alternative to staining procedures.

Antibiotics were identified by virtue of their localization site in the plate in terms of its corresponding standard. The amount of antibiotic in the chromatographic spot was estimated by visual comparisons with the pertinent standards.

The technique for performing the chromatographic procedure selected for determining antibiotics in anti-FMD vaccines is described in table 1. Also, the reagents and materials used (*17*) for this purpose are indicated in tables 2 and 3, respectively.

The standardized chromatographic procedure was then applied to determine the concentration of penicillin, polymyxin and neomycin in 10 lots of anti-FMD oil vaccine. Of these, seven were manufactured by commercial firms in Argentina and Colombia, while the remaining three lots had been prepared at the Pilot Plant for Vaccine Production, PANAFTOSA. Findings are shown in table 4.

## RESULTS AND DISCUSSION

In general, the values for polymyxin and penicillin recorded for all commercial vaccine lots analyzed were comparable to those of the vaccines prepared at PANAFTOSA. However, the situation with neomycin was different. Thus, although the three PANAFTOSA vaccines and six of the commercial immunogens fell within the same range of values, the neomycin level recorded for the remaining commercial vaccine (#3) was markedly higher. Findings were identical in repeated analyses.

Essentially, the above observations demonstrate that TLC is suitable for determining the levels of the antibiotics present in anti-FMD oil vaccines and suggest that this procedure may be

**Table 1. Chromatographic procedure for determining antibiotic levels in anti-foot-and-mouth disease oil vaccines**

**1. Extraction**

Test vaccines are allowed to reach room temperature and stirred for two minutes. Immediately, 3 ml are collected for extraction and introduced into a 10 ml tube having a ground glass stopper. After adding 6 ml of dichloromethane and 0.5 ml of water, tubes are stoppered, stirred for 3 minutes in a vortex, and allowed to rest for 3 minutes. Centrifugation is carried out at 2000 rpm for 3 minutes. The supernatant aqueous phase is transferred to a 5 ml tube using a pipette dropper. One ml of water is added to the oil residue and the procedure is repeated twice. All aqueous extracts of vaccines are pooled and concentrated to approximately 1 ml under a 60°C air current.

**2. Elution**

Place a collecting tube (#1) under the solid phase extraction column. Pour the aqueous phase concentrate obtained from the extractions into the column, regulating the vacuum applied so as to obtain a drop count of 1-2 drops per second. The receptable containing the extracts is washed with 0.5 ml of water, which is then poured through the column. Elution is carried out with methanol (table 2). Another collecting tube (#2) is placed under the column, eluted with 5ml of methanol, transferred to the next tube (#3) and eluted with 3 ml of acetone. Finally, the eluate is placed into the last collecting tube (#4), and eluted with 6 ml of the Elution Mixture II (table 2).

**3. Concentration**

The recipients containing the eluate of collecting tubes #2 and #4 are placed respectively in a water bath or air current at approximately 40°C. A smooth current of clean dry air is placed on its surface, stirring periodically as concentration proceeds, either manually or with a vortex, to wash the walls. The solution is allowed to concentrate until a 0.2 ml volume is reached.

**4. Thin layer chromatography**

The procedure to identify and evaluate the antibiotics extracted from vaccines is carried out as follows:

**4.1** ***Sample application and elution.*** Concentrates (5 µl) obtained from collecting tube #2, as well as the penicillin standards, are respectively placed on the reverse phase TLC plate, and then developed using Elution Mixture I (table 2). The same amounts of the concentrates obtained from collecting tube #4 and the neomycin and polymyxin standards are applied on chromatofolio with silicagel and developed with Elution Mixture II (table 2). In each case, a 1.5 cm separation is allowed between neighboring samples. These are then run 12 cm from the application spot, using the corresponding eluting mixture. TLC plates are then removed from the developing chamber for drying in a current of warm air.

**4.2** ***TLC Development.*** TLC plates for penicillin determinations are placed in a chamber with small amounts of iodine crystals and allowed to remain until yellow spots appear in the sites where standards were applied. Exposure to iodine vapors is then prolongued for two more minutes. Plates are removed and left exposed to air until only the spots are seen and the rest of the TLC plate remains white. To increase sensitivity, plates are sprayed with the starch solution. The purple spots which appear persist for several hours, while the yellow spots formed by iodine disappear within the hour.

TLC plates are sprayed with the ninhydrin solution (table 2) to determine polymyxin and neomycin and allowed to react for approximately 5 minutes in a 55°C oven, until purple stains appear in the sites corresponding to each antibiotic.

**5. Identification and evaluation**

Antibiotics are identified on the basis of their position in TLC plates vis-à-vis that of the corresponding standard. The amount of each antibiotic in the chromatographic spots is estimated by comparing visually spots formed by each sample of the vaccine extracts, with that of the given reference standard.

**6. Calculations**

The milligrams of antibiotic per milliliter of vaccine are determined by dividing the micrograms obtained in the preceeding stage by a factor of 75.

**Table 2. Reagents used for the determination of antibiotics in anti-foot and-mouth disease oil vaccines by thin layer chromatography***

Dichloromethane PA, methanol PA, acetone PA and ammonia PA are used.

*Elution Mixture I:* Methanol PA, 0.1 M dipotassium phosphate 0.1 M 6:4 solution.

*Elution Mixture II:* Methanol PA, acetone PA, chloroform, ammonia PA (c) 3:2:2:2.

*Developing solution:* Dissolve 0.30 g ninhydrin PA (Riedhl de Haen 33437) in 100 ml of N-Butanol. Subsequently, add 3 ml glacial acetic acid.

*Resublimated iodine*, (Cat. Fisher 135-100).

*Starch solution:* Soluble starch (Difco 0178-17), 1% in distilled water.

incorporated in the quality control of these immunogens on the basis of its sensitivity, reproducibility, specificity, simplicity and low cost. Furthermore, application of TLC to analyze the final product may provide reliable information which may be used to evaluate the production process. For instance, the detection of high antibiotic levels in vaccines may be indicative of the existence of contamination problems in the harvesting of the cellular substrate and/or at the stage of inoculating viral particles for preparing vaccine antigens. Solving these problems may also represent a savings in the expenditures represented by the addition of antibiotics during production of the immunogen, as well as in obtaining an end-product of better quality. Also, the availability of information on the antibiotics present in the different lots of vaccines applied to animals in the field offers the possibility of evaluating their relationship to the appearance of postvaccinal reactions, should they be reported.

Finally, the findings of the present study are also suggestive of the convenience of expanding the spectrum of antimicrobial agents which may be detected in anti-FMD vaccines by TLC to achieve an optimal quality control. Due to their association

**Table 3. Materials required for determination of antibiotic levels in anti-foot-and-mouth disease oil vaccines***

Tubes (10 ml) with ground glass stopper. Short 146 mm, Pasteur Pipette, (Fisher 13-678-70B). 5 ml test tubes. Mills tubes having 4 ml capacity, graduated in the lower portion at 0.1 ml (Kontes No. K 57 0050-0425).

Chromatographic columns: 7 mm interior diameter and 80 mm high, with coarse fritted plate. Alternative: use syringes with a filter paper disk in the lower portion to interfere with passage of Silicagel. Packed with 600 mg Silicagel, mesh 100-120 (Fisher 5679). Another option: commercially-prepared columns (Worldwide Monitoring Corp. SIL 153).

Micropipets of 5 µl or Hamilton (701 N) 10 µl syringes. TLC aluminum sheets Silicagel 60F254, sheet thickness 0,2 mm (Merck 5553). Reverse phase TLC plates n-octyl (Whatman 4808-820). Chromatography trays and sprayer. Hot bath or heater with the capacity to maintain between 30 and 60°C.

System for supply of air pressure. Device which permits supply of a dry preferably hot air current over the surface of the liquid to be evaporated (for example, stainless steel needles or Pasteur pipettes).

Oven that can be kept at 55 °C temperature.

* Mention of commercial firms or their products is for identification only and does not imply endorsement by the authors or their institutions.

**Table 4. Levels of three antibiotics detected in 10 foot-and-mouth disease oil vaccine lots manufactured by commercial firms and at PANAFTOSA***

| Vaccine No. | Product | Polymyxin | Penicillin | Neomycin |
|---|---|---|---|---|
| 1 | Commercial | 0.020 | 0.060 | ND |
| 2 | Commercial | 0.025 | 0.050 | 0.002 |
| 3 | Commercial | 0.025 | 0.050 | 0.140 |
| 4 | Commercial | 0.030 | 0.070 | 0.002 |
| 5 | Commercial | ND | 0.030 | ND |
| 6 | Commercial | 0.025 | 0.050 | 0.002 |
| 7 | Commercial | 0.035 | 0.070 | ND |
| 8 | PANAFTOSA | 0.020 | 0.040 | ND |
| 9 | PANAFTOSA | 0.028 | 0.085 | 0.003 |
| 10 | PANAFTOSA | 0.025 | 0.080 | 0.002 |

* Values for each concentration are expressed in milligrams of the antibiotic per milliliter of oil vaccine. ND = no detected.

with postvaccinal reactions in the past (*3,13, 14,15*), streptomycin and also merthiolate determinations in anti-FMD vaccines are considered of particular interest in our laboratories.

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## Information

South American Commission for the Control of Foot-and-Mouth Disease (COSALFA), composed of the Animal Health Directors of the South American countries, meets annually and serves as an advisory body to the PANAFTOSA Director. It is also the entity that promotes, coordinates and assesses the national programs, subregional projects and border agreements of the Region's countries as regards foot-and-mouth disease and other vesicular diseases.

In March, 1985, the Ministers of Foreign Relations of the South American countries institutionalized COSALFA as a Permanent Subregional Commission. The Foreign Relations Ministry of Brazil acts as the depository of the Agreement. COSALFA is also entrusted with adopting norms and measures to prevent the introduction of exotic diseases into South America.

## Announcement

**Meetings of the South American Commission for the Control of Foot-and-Mouth Disease (COSALFA)**

*Meetings to discuss matters related to the prevention and control of foot-and-mouth disease are held annually by the member countries of COSALFA. Prior to each meeting, a Seminar on a topic selected during the preceding Seminar is also held.*

1993 - *XX Regular Meeting of COSALFA* . March 25 and 26, 1993, Montevideo, Uruguay
International Seminar on Foot-and-Mouth Disease Eradication, its Technical and Administrative Bases and its Consequences on the Commercialization of Animals and their Products and by-products. March 22 to 24, 1993.

1994 - *XXI Regular Meeting of COSALFA*. April 14 and 15, 1994, Lima, Peru
International Seminar on the Animal-Health Care Systems and the Changes in the Role of the State and the Community. April 11 to 13, 1994.