TREATMENT OF VIRUS SUSPENSIONS WITH TRICHLOROTRIFLUORETHANE FOR THE PRODUCTION OF FOOT-AND-MOUTH DISEASE VACCINE ON AN INDUSTRIAL SCALE

G.C. DARSIE[1], R.G. CUNHA[2], I. GOMES[1]

[1]Pan-American Foot-and-Mouth Disease Center (PAHO/WHO)
P.O. Box 589, 20001-970 Rio de Janeiro, RJ, Brazil
[2]Faculdade de Veterinária, Universidade Federal Fluminense
Rua Vital Brazil Filho, 64, 24220 Niterói, RJ, Brazil

Summary. **Suspensions of foot-and-mouth disease virus were treated on an industrial scale with 1% v/v of trichlorotrifluorethane (TTE) or chloroform to determine the viability of TTE as an alternative agent for the partial purification of vaccine antigens. The suspensions treated with chloroform showed an average reduction of 50.4% of the total protein content, while the suspensions treated with TTE showed a reduction of 33.4%. The vaccines formulated with the antigens treated with either of the two solvents did not present significant differences in the induction of immunity when assessed by the expected percentage of protection (EPP) of young cattle vaccinated for the first time. No disadvantages were noted in handling the TTE, nor were there any undersirable postvaccination reactions.**

The foot-and-mouth disease (FMD) virus is a representative of the Picornaviridae family, whose seven serotypes compose the Aphthovirus genus. Its virion is formed of a simple ribonucleic acid chain coated by a lipid-free protein capsid (*3*) which renders it resistant to organic solvents like chloroform (*26*) and the fluorocarbons (*16*).

FMD vaccines, applied jointly with other measures, have been crucial in the fight to control the disease. The antigens are produced from *in vitro* cell cultures in which the virus replicates. Due to the lysogenic characteristic of its replication cycle, the virus, at the end of the culture, kills the cells releasing large amounts of cell proteins, lipids and other components into the medium, which if not removed, are incorporated as antigens into the vaccine. For this reason, it is commom practice to partially purify viral suspensions using 1% chloroform, by shaking or using a homogenizer, followed by clarification and filtration or continuous centrifugation (*7*).

Although efficacious, the use of chloroform poses a problem due to the turbidity of suspensions obtained from cell cultures that utilize bovine serum treated with polyethyleneglycol (PEG) (*1*); also undesirable is the wear it causes in equipment, the risks of accidents due to acute intoxication and the growing difficulties encountered in acquiring chloroform in some countries because of its illegal use as a drug purifier.

The search for a solvent to replace chloroform as a purifying agent in production of viral suspensions on an industrial scale revealed the possibility of using trichlorotrifluorethane (TTE). No references related to the stability of the antigens during storage were found, nor that of the oil-

Reprint requests to:
Pan-American Foot-and-Mouth Disease Center (PAHO/WHO).

adjuvanted vaccines produced with those antigens or about the possible occurrence of undesirable postvaccination reactions in cattle.

This study presents the results obtained in the comparison of TTE with chloroform, plus those related to the significant reduction of the concentration used.

MATERIALS AND METHODS

Nine monovalent suspensions each of 300 liters of volume were prepared as part of the routine antigen production at the Vaccine Production Plant of The Pan American Foot-and-Mouth Disease Center. Five suspensions were O_1 Campos-Br/58 strain (OCAM); two were A_{24} Cruzeiro-Br/55 strain (ACRU); one was A-79 Venceslau-Br/76 strain (AVEN); and one was C_3 Indaial-Br/71 strain (CIND).

$BHK_{21}C13$ cells were cultivated in 120 to 600 litre tanks. Eagles medium containing 8% PEG-treated cattle serum, sterilized by filtration in a 0.22 µm cartridge (*8*) was used. After sedimentation of the cells, the virus growth medium was added. Those cultures intended for production in roller bottles were diluted to a concentration of 0.2 x 10^6 cell/ml in Eagles medium plus 8% of untreated serum, and the medium was drawn off after 72 hours of growth.

The cell cultures were inoculated with virus seeds at a rate of 0.02-0.06 infective viral particles per cell in serum-free Eagle medium containing a final concentration of 30% of 0.2M Tris-hydroximethylaminomethane solution.

At the end of virus replication, the suspensions were chilled at 4 °C. Volumes of 10 liters were treated with 1% of chloroform using magnetic bar agitation in glass vessels for 30 minutes. The remainder was treated with 1% TTE using a closed circuit homogenizer, at 3500 rpm for one hour. After 18 hours of standing at 4°C all of the supernatants were centrifuged at 7680 g.

Virus suspensions were inactivated with binary ethylenimine (*6*) at a final concentration of 3mM for 24 hours at 26 °C. The inactivant was hydrolyzed with 1M sodium thiosulfate just before the antigens were processed further.

During production the entire process was controlled by routine biological and physicochemical assays (*14*). In order to evaluate the efficiency of the treatments, the total protein content of some suspensions was determined prior to addition of the solvent and after clarification (*9*).

Eighteen paired vaccines were prepared with the nine monovalent antigens treated with TTE or chloroform, for tests of potency, innocuity, and antigenic stability after 300 days' storage at 4 °C. Volumes of two monovalent OCAM antigens and of two monovalent ACRU antigens were stored for 240 days at 4 °C and, after that period, eight pairs of vaccines were produced for potency testing.

All the vaccines were formulated with Freund's incomplete adjuvant (*2,15*) as per methodology described by Abaracón *et al* (*2*).

The influence of the treatments on immunogenicity was assessed by means of the expected percentage of protection (EPP) test, based on the indices of antibodies in the serum of primovaccinated cattle as determined by seroprotection in suckling mice or serum neutralization in cell culture (*24,28*).

All the results were analysed statistically by means of the Tukey test or by variance analysis with $P > 0.05$ (*24, 28*).

RESULTS

This study presents the findings referring to the action of TTE as an agent for partial purification in comparison with chloroform. Table 1 presents the results regarding partial purification of the suspensions under study.

TABLE 1. Total protein content (mg/ml) in viral suspensions treated with 1% of trichlorotrifluorethane (TTE) or chloroform (CLO).

	Total proteins (mg/ml)			% Reduction	
Suspension	Untreated	TTE	CLO	TTE	CLO
A	0,41	0,28	0,20	31,7	51,2
B	0,35	0,23	0,18	34,3	48,6
C	0,45	0,30	0,22	34,0	51,5

These values indicate that there was an average total protein content of 33.4% in the suspensions treated with TTE and 50.4% in those treated with cloroform; this difference is statistically significant.

Table 2 shows the results obtained with the 18 paired vaccines tested immediately following their production (0 DPP).

TABLE 2. Values of the lower limit of reliability of expected percentage of protection (EPP) achieved by the vaccines formulated with recently produced antigens (zero DPP), treated with trichlorotrifluorethane (TTE) or chloroform (CLO).

Vaccines		EPP "ZERO" DPP	
		TTE	CLO
OCAM*	1599	78,7	87,5
OCAM	1602	91,3	93,0
OCAM	1605	85,0	84,6
OCAM	1606	89,6	86,8
OCAM	1607	96,1	95,6
ACRU	1613	83,2	88,4
ACRU	1622	96,4	94,6
AVEN	1604	84,3	90,8
CIND	1603	98,5	99,0

*OCAM-O1 Campos-Br/58; ACRU-A24 Cruzeiro-Br/55; AVEN-A-79 Venceslau-Br/76; CIND-C3 Indaial-Br/71.

It can be seen that the immunogenic responses very slightly with the treatment but does not indicate a predominant influence of the treatments. The results shown in Table 3 for the eight vaccines stored at 4 °C for 300 days (300 DPP) show a similar relationship between the two treatments although there is an overall drop in immune response with respect to the data obtained with Zero DPP.

The results for the vaccines produced with the antigens stored at 4 °C for 240 days (240 DPP) also indicate a decline in the immune response (Table 4) with respect to zero DPP, although with less intensity than the results noted at 300 DPP.

The variance analysis applied to the results reveal significant differences in the EPPs found in the various times studied, as well as among the

TABLE 3. Values of the lower limit of reliability of expected percentage of protection (EPP) reached by vaccines stored at 4 °C for 300 days post-production (300 DPP), with antigens treated with trichlorotrifluorethane (TTE) or chloroform (CLO).

Vaccines		EPP 300 DPP	
		TTE	CLO
OCAM*	1599	67,5	65,6
OCAM	1602	81,5	83,3
ACRU	1613	80,5	83,9
ACRU	1622	82,4	86,1

*OCAM-O1 Campos-Br/58; ACRU-A24 Cruzeiro-Br/55.

strains utilized. There was no significant difference between the immune responses induced by the vaccines formulated with antigens treated with TTE or chloroform.

622 calves were vaccinated in the experiment and in no case were undesirable postvaccination reactions noticed.

In practice, it was noted that there was a reduction in the time required to sediment cell debris and that no turbidity occurred in the suspensions treated with TTE, as compared to those treated with chloroform. Nor were any disadvantages noticed with regard to their handling, such as toxic odors or damage to the equipment.

Table 4. Values of the lower limit of reliability of expected percentage of protection (EPP) reseached by vaccines formulated with antigens treated with trichlorotrifluorethane (TTE) or chloroform (CLO) and stored at 4 °C for 240 days post-production (240 DPP).

Vaccines		EPP 240 DPP	
		TTE	CLO
OCAM*	1599	77,8	89,0
OCAM	1602	79,1	79,9
ACRU	1613	80,4	80,9
ACRU	1622	90,0	83,0

*OCAM-O1 Campos-Br/58; ACRU-A24 Cruzeiro-Br/55.

DISCUSSION

This study found an average reduction of 33.4% of the protein content in FMD virus suspensions when they were treated with only 1% TTE in a single cycle. The bibliography consulted revealed information on the high degree of purification of viral suspensions which could be achieved, but by using up to 50% of chlorofluorocarbons (*16*), and a high number of cycles of solvent treatment and centrifugation (*14*). The excellent performance described can be attributed to the mixing process using a high potency homogenizer that provided better solvent dispersion and interaction. It should be noted that Manson *et al.* (*23*) obtained similar results in studies with poliovirus after two cycles at a proportion of 50%.

The use of liquid fluorocarbons in virus preparations began with Gessler (*16*), who purified smallpox and Rous sarcoma virus suspensions. Other procedures were developed to purify poliomyelitis virus, adenovirus and T5 coliphage (*23*), to eliminate anticomplementary factors of Coxsackie (*18*) and poliomyelitis viruses (*20*), and for the dissociation of complexes formed by poliomyelitis virus and antibodies (*19*).

TTE is a colorless, nonflammable, liquid chlorofluorocarbon, with a low degree of toxicity, utilized in the electronic industry as a cleaning agent for high-tech components. There are references about its use in the purification of FMD virus for electronic microscopic identification of viral particles (*5*), as well as in studies of complement fixation activity (*25*), the dissociation of virus-antibody complexes (*29,31*), purification for concentration by PEG (13), and for the production of vaccines of different types (*4,10,14,21,22,27*).

The TTE showed a single disadvantage in comparison with the treatment using chloroform, namely a reduction in the level of protein removal although this situation can be lessened or even reversed by using a greater proportion of TTE or a larger number of treatment cycles. With respect to the immune response induced by the vaccines tested, the results indicate that there was no diminution of the antigenic capacity of the suspensions treated with TTE as compared to those treated with chloroform. This confirms the findings of other authors (*10,14,22,27*), and reaffirms the possibility of TTE being used as an alternative in the industrial treatment of FMD virus suspensions.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Prof. Elizabeth B.B. Pereira, to Drs. Raúl Casas Olascoaga, Vicente M. Astudillo, Ubiratan Mendes Serrão and Alberto Knust Ramalho, for their generous assistance and cooperation.

This study was submitted to the Federal Rural University of Rio de Janeiro, Brazil, in partial fulfillment of the requirements for the degree of *Magister Scientiae* in Veterinary Pathology in the area of Preventive Veterinary Medicine.

REFERENCES

1. ABARACON, D., GIACOMETTI, H. Vacunas contra la fiebre aftosa con virus producido en cultivos celulares con suero bovino tratado por polietilenoglicol. *Bol. Centr. Panam. Fiebre Aftosa, 21-22*: 44-48, 1976.
2. ABARACON, D., MESQUITA, J.A., SALLUA, S., PEREZ RAMA, R. Emulsificante montanide 888 para la preparación de vacunas antiaftosa con adyuvante oleoso. *Bol. Centr. Panam. Fiebre Aftosa, 45-46*: 51-53, 1982.
3. ANDREWES, C.H. *Viruses of vertebrates*. 5ª ed., London, 1989. p. 121-143,
4. AUGÉ DE MELLO, P., ASTUDILLO, V., GOMES, I., CAMPOS GARCIA, J.T. Aplicación en el campo de vacuna antiaftosa oleosa y inactivada: vacunación y revacunación de bovinos jóvenes. *Bol. Centr. Panam. Fiebre Aftosa, 19-20*: 31-37, 1975.
5. BACHRACH, H.L., BREESE, S.S. Purification and electron microscopy of foot-and-mouth disease virus. *Proc. Soc. Exp. Biol. Med., 97*: 659-665, 1958.
6. BAHNEMANN, H.G. Binary ethylenimine as an inactivant for foot-and-mouth disease virus and its application for vaccine production. *Arch. Virol., 47*: 47-56, 1975.

7. BAHNEMANN, H.G., MESQUITA, J.A. Vacuna antiaftosa con adyuvante oleoso. *Bol. Centr. Panam. Fiebre Aftosa, 53*: 19-24, 1987.
8. BAHNEMANN, H.G., MESQUITA, J.A., ASTUDILLO, V., DORA, F. The production and application of an oil adyuvant vaccine against foot-and-mouth disease in cattle. In: *8th Meet. Europ. Soc. for Anim. Cell Technol. Modern Approaches to Animal Cell Technology*. Butterwoths, Butterworth e Co., 1987. p. 628-639.
9. BRADFORD, M.M. Rapid scientific methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem., 72*: 248, 1976.
10. BROWN, F., CARTWRIGHT, B. Purification of the virus of foot- and-mouth disease by fluorcarbon treatment and its dissociation from neutralizing antibody. *J. Immunol., 85*: 309-313, 1960.
11. CENTRO PANAMERICANO DE FIEBRE AFTOSA. *Manual de procedimientos para el control de vacuna antiaftosa*. Rio de Janeiro, PANAFTOSA, 1980. 47 p. (Ser. Man. Técnicos, 2).
12. CUNHA, R.G., BAPTISTA JR., J.A., SERRÃO, U.M., TORTURELLA, I. El uso de los ratones lactantes en la evaluación de los anticuerpos contra el virus de la fiebre aftosa y su significación inmunológica. *Gac. Vet., 19*: 243-267, 1957.
13. FAYET, M.T. Concentration du virus de la fièvre aphteuse par le polyéthylène glycol. *Ann. Inst. Pasteur, 118*: 356-366, 1970.
14. FAYET, M.T., ROUMIANTZEFF, M., DUBOUCLARD, C., FONTAINE, J. Utilisation d'un fluorocarbone comme méthode d'étude du virus de la fièvre aptheuse. *Ann. Inst. Pasteur, 109*: 652-662, 1965.
15. FREUND, J., THOMPSON, K.M. A simple rapid technique of preparing water in oil emulsion of penicilin, drugs and biologicals. *Science, 101*: 468-469, 1945.
16. GESSLER, A.E. A new and rapid method for isolating viruses by selective fluorocarbon deproteinization. *Trans. N.Y. Acad. Sci., 18, Ser. II*, (8): 701-703, 1956.
17. GOMES, I., ASTUDILLO, V. Foot-and-mouth disease: evaluation of mouse protection test results in relation to cattle immunity. *Bol. Centr. Panam. Fiebre Aftosa, 17-18*: 9-16, 1975.
18. HAMPARIAN, V., MÜLLER, F., HUMMELER, K. Elimination of nonspecific components from viral antigens by fluorocarbon. *J. Immunol., 80*: 468-475, 1958.
19. HUMMELER, K., KETLER, A. Dissociation of poliomyelitis virus from neutralizing antibody. *Virology, 6*: 297-299, 1958.
20. HUMMELER, K., HAMPARIAN, V. Removal of anticomplementary activity and host antigens from viral preparations by fluorocarbon. *Science, 125*: 547-548, 1957.
21. KLIMOV, N.M., MALAKHOV, A.G., GRIBANOV, V.N. Chemical purification of lapinized foot-and-mouth disease virus and trials of its antigenic and immunogenic properties. *Trudy Vsesoyus. Inst. Eksp. Vet., 24*: 208-214, 1961.
22. MACKOWIAK, C., FONTAINE, J. Utilisation de virus traites au fluorocarbone pour la préparation du vaccin antiaphteux. In: *XVIII Congrès Mondial Vétérinaire*, Paris, 17-22 Jul. 1967. p. 383-386.
23. MANSON, L.A., ROTHSTEIN, E.L., RAKE, G.W. Purification of poliovirus with fluorocarbon. *Science, 125*: 546-547, 1957.
24. PIMEMTEL GOMES, F. *Curso de estatistica experimental*. Piracicaba, SP, USP/ESALQ, 1971, 621 p.
25. POLATNICK, J. Studies on the small particle complement-fixing antigen of foot-and-mouth disease virus. *Proc. Soc. Exp. Biol. Med., 103*: 27-31, 1960.
26. PYL, G. Chloroform treatment of brain material infected with neurotropic viruses. *Exp. Vet. Med., 5*: 1-5, 1951.
27. ROUMIANTZEFF, M., FONTAINE, J., DUBOUCLARD, C. Évaluation du pouvoir immunogène du virus aphteux par mesure du pouvoir fixant le complément après traitement par un fluorocarbone. *Compt. Rend. Acad. Sci., 261*: 598-601, 1965.
28. SNEDECOR, G.W., COCHRAN, W.G. *Statistical methods*. 6ª ed. Ames, Yowa State Univ., 1972. 593 p.
29. SUTMÖLLER, P., COTTRAL, G.E. Improved techniques for the detection of foot-and-mouth disease virus in carrier cattle. *Arch. ges. Virusforsch., 21*: 170-177, 1967.
30. SUTMÖLLER, P., GOMES, I., ASTUDILLO, V. Estimación de potencia de vacunas contra la fiebre aftosa de acuerdo con los resultados de pruebas de anticuerpos. *Bol. Centr. Panam. Fiebre Aftosa, 49-50*: 27-30, 1984.
31. TESSLER, J. Reactivation of antibody-neutralized foot-and-mouth disease virus by organic chemicals and inhibition by 1-butanol. *Am. J. Vet. Res., 119*: 917-922, 1966.