AGA:DP/TUR/69/533
Technical Report 1

# ASSISTANCE TO THE FOOT AND MOUTH DISEASE INSTITUTE, ANKARA, FOR THE PRODUCTION OF VACCINE AND THE TRAINING OF PERSONNEL

# TURKEY

## VACCINE PRODUCTION

UNITED NATIONS DEVELOPMENT PROGRAMME

FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS ROME, 1972

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T U R K E Y

## VACCINE PRODUCTION

Report prepared for
the Government of Turkey
by

the Food and Agriculture Organization of the United Nations
acting as executing agency for
the United Nations Development Programme

based on the work of

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UNITED NATIONS DEVELOPMENT PROGRAMME
FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 1972

This technical report is one of a series of reports prepared during the course of the UNDP/SF project identified on the title page. The conclusions and recommendations given in the report are those considered appropriate at the time of its preparation. They may be modified in the light of further knowledge gained at subsequent stages of the project.

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ABSTRACT

The Government of Turkey, with the assistance of the United Nations Development Programme and the Food and Agriculture Organization of the United Nations, implemented a project at the Foot-and-Mouth Disease Institute, Ankara, in November 1969. The major objectives of this project were to introduce techniques to substantially increase the growth of Foot-and-Mouth Disease (FMD) virus for the production of vaccines; to develop methods which would lead to the standardization of the vaccines so produced; to examine methods for the differentiation of types and sub-types so that the appropriate strains of virus could be used for vaccine production; and to engage in research which would materially assist in the basic aims of the project.

One of the most important methods in growing FMD virus is the use of baby hamster kidney (BHK) cells suspended in a suitable liquid medium. Techniques involving this new method were successfully introduced for the large scale industrial production of vaccine. Two new methods of testing vaccines for potency were developed: adult mice were vaccinated and the antibody response was measured; and the virus was physically separated into its immunogenic and antigenic fractions and the two components were critically measured.

Typing of the virus from recent field outbreaks of FMD showed the presence of a strain which varied sufficiently from the usual type A to warrant attention in formulating control programmes based on vaccination.

As it is now possible to substantially increase production of FMD virus suitable for vaccines, continuing care must be taken to ensure that the vaccines are potent and are derived from the types and sub-types currently causing outbreaks in the field. To achieve this it is recommended that the work now reported be strengthened and expanded and that additional national staff be trained in the new methods.

The Food and Agriculture Organization of the United Nations is greatly indebted to the organizations and individuals who assisted in the implementation of the project by providing information, advice and facilities.

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# Chapter 1

# INTRODUCTION

## 1.1 PROJECT BACKGROUND

Turkey, with a livestock population estimated at 13 million cattle and 50 million sheep and goats, is particularly vulnerable to the introduction of exotic disease. The country has been invaded twice through its eastern borders by exotic types or sub-types of Foot-and-Mouth Disease (FMD); South African Type 1 (SAT 1) in 1962 and A 22 virus in 1964. While in both cases it was possible, through systematic vaccination, to prevent the spread of the disease towards Europe, each time it was too late to protect the Turkish livestock which suffered considerable damage. Apart from the actual losses, the introduction of exotic viruses harmed the livestock industry by excluding existing and potential markets in Europe for Turkish livestock and animal products.

The geographical position of the country between Europe and the Near East deserves particular consideration. The invasion of Europe by exotic strains of FMD would cause incalculable losses, because the regular vaccination of European livestock against the usual types A, O and C would fail to protect the animals against the SAT 1 and A 22 viruses, or the Asia 1 and SAT 2 viruses which are known to have invaded other countries in the region.

In view of these threats, and so that research aimed at the control and eventual eradication of FMD could be expanded, the Government of Turkey provided over US $ 1 000 000 for the construction of new laboratory facilities. The European Economic Community, well aware of the dangerous situation, contributed US $ 500 000 for laboratory equipment.

It was estimated that Turkey could produce only 1 500 000 trivalent doses of Waldman, Frenkel and cattle kidney cell tissue culture type vaccines annually against the three common FMD types found in the country. As 10 000 000 doses are required each year it was realized that newly developed production methods, yielding greatly increased amounts of vaccine, would have to be introduced.

Production of vaccine, on the scale envisaged, is a highly technical and complex operation requiring staff skilled in many disciplines. The Turkish Government accordingly invited the United Nations Development Programme (Special Fund) to assist, by recruiting experienced international staff to collaborate with their own scientists in a programme of research aimed at solving some of the problems in FMD control.

## 1.2 OUTLINE OF OFFICIAL ARRANGEMENTS

The project was authorized by the Governing Council of the United Nations Development Programme in January 1969 and the Food and Agriculture Organization of the United Nations was designated executing agency with the Ministry of Agriculture, Division of Veterinary Services, Foot-and-Mouth Disease Institute in Ankara as the counterpart agency.

## 1.3 OBJECTIVES OF THE PROJECT

The aim of the project was to assist the Government of Turkey to increase and improve facilities and train national staff in large-scale production of FMD vaccines.

In particular the project would assist by:

(a) developing methods for the use of baby hamster kidney (BHK) cell lines in suspension for the propagation of virus for vaccines;

(b) investigating the possibility of increasing vaccine production by using the Frenkel double culture method;

(c) installing modern equipment for the dispensing and bottling of vaccines;

(d) installing an automatic system for the measurement of antigenetic titres, required because of increased vaccine production due to enhanced virus growth;

(e) developing facilities for the rapid and accurate identification of types and sub-types of FMD virus responsible for outbreaks of disease in Turkey so that vaccines could be prepared using the appropriate strains; in the event of outbreaks of FMD occurring in neighbouring countries and presenting a threat to Turkish livestock the project would, with the prior approval of the Turkish Government, receive material from those countries so that the virus types and sub-types could be determined;

(f) furthering research on the physical and chemical properties of the virus of FMD to evaluate their potential immunogenic properties;

(g) selecting and installing, calibrating, operating and maintaining specialized equipment;

(h) emphasizing the training of national staff in all aspects of project work and providing training opportunities for professional and technical staff from other countries in the region.

# Chapter 2

# APPRAISAL OF ACCOMPLISHMENTS

## 2.1 VACCINE PRODUCTION

### 2.1.1 Introduction

The growth of FMD virus occurs only in living cells and replication of the virus, in quantity, is one of the fundamental problems in large scale vaccine production. Three basic methods for the propagation of FMD virus are available, the Waldmann, the Frenkel and tissue culture.

Waldmann's method involves the injection of the virus into the tongue of healthy cattle; the animals are slaughtered 24 hours later and the tongue epithelium, rich in virus, harvested. In Frenkel's method healthy cattle are slaughtered and the tongue epithelium is harvested. The harvest is suspended in a medium, capable of supporting cell life, and infected with the selected virus. The usefulness of both methods is restricted by the numbers of normal cattle available and the fact that the carcasses, in the Waldmann method, are used for human consumption thus presenting a serious threat of spreading the disease.

Tissue culture (TC), the third method, makes use of kidney cells from normal animals. The cells grow as monolayers in special flasks. Once growth is well established, usually after six to seven days in the case of calf kidney cells, virus is introduced into the flasks and replication takes place. But, here again, it is difficult to secure a constant supply of normal calf kidneys. To obviate this difficulty, baby hamster kidney (BHK) cells were used, first as monolayers in flasks and later as cellular suspensions in liquid medium.

Although this brief outline of the basic methods for virus replication suggests that the growth of FMD virus is a simple process, the actual technical requirements to achieve successful large-scale production are complex. The problems were studied in two separate phases; the growth of normal BHK cells in suspension and the replication of the virus.

### 2.1.2 Growth of BHK Cells in Suspension

BHK cells in suspension provide a suitable means for the large-scale growth of FMD virus and they have several special properties. The normal BHK cell can be replicated indefinitely in the laboratory, in contradistinction to calf kidney cells which can only be grown over a limited number of generations; they have been adapted to grow in suspension and do not require a supporting background as is usual with many cell lines used in TC work. As it is an established cell line it can be stored

indefinitely in the laboratory thus doing away with the expensive acquisition of normal cattle; BHK cells in suspension require the use of laboratory techniques which are less complex and which are more adapted to commercial production methods than those used in monolayer procedures.

Although basic equipment was available, which was suited to the growth of cells in suspension, much of it had to be adapted and modernized. The fundamental unit in large-scale production of cells is a special tank - the 150 litre New Brunswick tank - and those available were successfully modified to improve their heating mechanism and adapt them for the continuous growth of cells. As a result of the modifications the yield of cells was more than doubled.

The experimental work for the replication of BHK cells in suspension began when the laboratory for TC work opened in March 1971. By the middle of the year, substantial progress had been made and in April 1972 satisfactory results from the modified tanks were achieved. Technical data are given in Appendix 1, Sections 1.1 to 1.4.

### 2.1.3 Replication of FMD Virus in BHK Cells in Suspension

While the project team had access to published information and the advice of consultants on the growth of normal BHK cells in suspension, little information was available, either in published form or from consultants, on the growth of FMD virus in the cells so produced.

Growth of the virus was by far the most difficult problem to be solved and involved the following:

(a) adaptation of the virus to BHK cells in monolayers and in suspension;

(b) selection of the best virus for experimental work;

(c) determination of the optimal pH and temperature for virus growth;

(d) a study of the kinetics of virus replication;

(e) selection of the virus capable of producing the highest antigen titres;

(f) correlation of the best immunizing virus with the most potent vaccine by challenge experiments using mice and cattle.

Before large-scale vaccine production could begin, many laboratory experiments had to be carried out. There are numerous types and sub-types (strains) of FMD virus and for each one it is necessary to determine the optimum time to harvest the primary cultures to be used as infective seed for further propagation. In addition, for each strain, it is necessary to determine if it is a satisfactory immunogen in the quantitative sense and at what stage of its growth in the BHK cell suspension it should be harvested to give a maximum yield of antigen.

Although some virus work began in July 1970 it was not until the second laboratory for virus research and analysis was completed in January 1971 that systematic research began. By April 1971, and following 26 major modifications, equipment was in regular use for the growth of virus on BHK cells in suspension

in tanks on a commercial scale. The modified culture tanks and ancillary equipment are the prototypes for the first steps in large-scale industrial production. Whilst they are working satisfactorily, additional modification may possibly increase their efficiency. Technical data are given in Appendix 1, Section 1.5.

### 2.1.4 FMD Virus Production Using the Frenkel Double Culture Method

Apart from virus propagation in BHK cells the most useful of the alternative methods is one developed by Frenkel. The original Frenkel method was modified by French workers so as to gain two harvests of virus from one batch of epithelial cells which greatly increased the potential for vaccine production. Basically the technique takes advantage of the fact that, as the epithelial cells are not single but are present in aggregation, not all cells are infected at the first seeding and harvesting of the virus.

Although the method was tried in Turkey several years ago results were irregular. As the available installations were not sufficient for modern working, new equipment was installed in the reorganized Frenkel production laboratory in June 1971. The new equipment, modelled on the French system, comprised laboratory and commercial production units. These are essentially the same in design so that experience gained on the laboratory unit can be applied readily to commercial production.

Production began in the new units in September 1971 but much remains to be done before the system can make a useful contribution to the supplies of vaccine.

### 2.1.5 Dispensing and Bottling of Vaccines

Increased availability of virus for vaccine production necessitated a reorganization of facilities for its inactivation, in appropriate tanks, and the provision of equipment for the preparation of polyvalent vaccines and the bottling of the end products. In view of the potentially large demand for vaccines these procedures were planned on modern lines of automation and the necessary equipment was ordered toward the end of the project.

## 2.2 RESEARCH ON FMD VIRUS

### 2.2.1 Quantitative Measurement and Components of FMD Virus

The measurement of virus growth is an important aspect in the preparation of vaccines. The presence of FMD virus may be detected using cattle or other experimental animals such as guinea pigs or unweaned mice and quantitative measurements can be made. Cattle are expensive and tests in animals may not always be conclusive. For example, it is known that guinea pigs do not always react and experience in Turkey showed that the local strains of the $O_1$ virus, grown in BHK cells in suspension, did not produce classical reactions in unweaned mice. Tissue culture techniques can also be used to detect the presence of virus; evidence of its presence is obtained when the monolayer of cells is destroyed - the so-called cytopathogenic effect. When the virus was grown in BHK cell suspensions, the reduction in cell numbers also provided a good indication of the presence of the infecting agent.

Two indirect quantitative tests have been developed to measure virus production. They are the infectious titre (IT) and the antigen titre (AT) tests.

2.2.2 Determination of the IT Using the Plaque Forming Unit PFU Test

For the accurate determination of the IT the plaque forming unit (PFU) test was used. This sensitive test is based on the fact that individual virus particles infecting a cell monolayer destroy the cells in centres or plaques when the spread of the virus is inhibited by a layer of solid or semi-solid agar medium overlaying the monolayer. When the monolayer is subsequently stained the plaques appear as round transparent areas which can be counted either macroscopically or microscopically, depending on their size. The number of PFU's present, and therefore the IT of a sample, can be calculated with a high degree of accuracy using simple mathematical formulas.

In Turkey, the original method was modified by using semi-solid methylcellulose instead of agar and replacing Petri dishes with test tubes as culture containers. By so doing, better use was made of the available incubator space and carbon-dioxide incubators were not necessary.

Two counterparts were trained in the technique and a large number of virus samples, originating mainly from BHK suspension cultures, was tested. Technical data are given in Appendix 2.

2.2.3 Plaque Reduction Tests

Counterparts were also trained in the plaque reduction (PR) test. This test, based on serum-neutralization, is more reliable than the indirect complement-fixation (CF) test, or the agar-gel diffusion test, for the detection of small quantities of specific antibodies in the sera; either the virus or the serum or both can be tested in serial dilutions.

Plaque reduction tests showed that practically all bovine sera, collected in 1970 from cattle in Thrace, which had previously been negative in indirect CF tests performed both manually and with the automated technicon did, in fact, contain neutralizing antibodies, some in relatively high concentration. Large quantities of neutralizing antibodies were also detected in serum pools obtained from the slaughter-house for use in growth media for BHK cells in suspension and monolayer cultures. As it is essential to eliminate these antibodies from the BHK cellular suspensions used to produce virus for vaccines, the test is of great importance to detect their presence.

Further PR tests were carried out in parallel, using sera from young cattle from the Antalya area, where periodic vaccination is not practised, and a negative bovine serum (control) from the U.S.A., where FMD does not exist. The sera from Antalya were completely free from both Types O and A neutralizing antibodies. Technical data are given in Appendix 2.

2.2.4 Determination of the Antigenic Titre (AT)

It is known that FMD virus has two components; the infectious and immunogenic particle of 22 mu and the antigenic particle of 7 mu. These may be separated by the chemical action of arcton, and the arcton treated antigenic titre (ATAT) is used to measure the potency of inactivated FMD vaccines.

It was found in practice, however, that the ATAT test on vaccines produced from BHK cell suspension did not always correlate with potency tests carried out on living animals. In some cases the BHK vaccine was shown to be a good immunizing agent, but the ATAT test was negative.

To overcome this problem a technique was developed to separate the two component particles by physical means, using ultracentrifugation, instead of the classical chemical method of extraction by arcton.

The Review Mission (May 1971) noted, with satisfaction, that zonal rotor ultracentrifugation had been introduced for fractionation of FMD antigens and stated that the techniques being introduced would place Ankara in the forefront of vaccine producing laboratories in this respect.

The work was, unfortunately, interrupted by the untimely departure of the expert. To complete the research already initiated it will be necessary to determine the quantity of the immunogenic component, extracted by ultracentrifugation, necessary to establish full protection in cattle. Technical data are given in Appendix 3.

2.2.5 Potency Testing of Vaccines Using the Complement Consumption Test

An indirect method of testing the potency of FMD vaccines in mice, the complement consumption (CC) test, was developed by Dr. E. Traub, one of the project's experts, and his co-workers in Germany in 1969.

The technique was used to assess the value of Type O vaccines produced in Turkey and results showed that the CC test, in general, agreed with vaccine potency trials in cattle.

While the test presented no problems when Frenkel type and BHK monolayer vaccines were used, difficulties arose in testing vaccines prepared from BHK cells in suspension. Investigations showed that the difficulties probably arose because of the method used in the preparation of the BHK cell suspension antigen. Further detailed work was carried out and, although the CC testing of BHK suspension antigen is much more laborious, there is no doubt that the new test is a useful laboratory method for determining the potency of vaccines.

The CC test was also successfully used in experiments:

(a) designed to remove an inhibitor of immunization present in Type O Frenkel vaccines;

(b) on the adsorption of infectious virus;

(c) on complement fixing and immunizing antigens prepared with locally produced aluminium hydroxide;

(d) to study the relative immunizing power of vaccines made from Type O virus and causing large and small plaques in BHK monolayers.

Technical data are given in Appendix 4.

2.2.6 Automation in Quantitative Measurement

National staff were trained in the use of a technicon auto-analyzer. The Review Mission noted that the successful introduction of the technicon, for the titration of antigen and serum antibodies, makes the Ankara laboratory only the second institute in the world to employ this rapid and highly accurate technique in FMD research.

## 2.3 IDENTIFICATION OF TYPES AND SUB-TYPES OF FMD VIRUS

It is important that the Turkish veterinary authorities receive immediate and reliable information of the presence of FMD, and the types and sub-types of the infecting virus, when outbreaks occur in bordering countries and especially in the Northern Provinces of Iraq and Syria. It was envisaged, in drafting the Plan of Operation, that the necessity might arise to study virus types existing in neighbouring countries. The Plan of Operation specified, however, that no such studies should be carried out without the approval of the Turkish Government.

To eliminate the danger inherent in permitting the importation of viable material, plans were evolved which would permit a reliable examination to be made on inactive and safe samples. These plans involved the training of the NEAHI epizoologist in the FMD Institute, Ankara on the methods of collecting and inactivating the suspect samples at the point of collection so as to render them completely harmless.

Now that a method to protect Turkey against the accidental release of foreign virus strains is available, it is confidently hoped the Turkish Government will grant permission for the receipt of suspect samples from Northern Iraq and Syria. Examination of the samples in the modern and fully-equipped laboratories of SAP Enstitusu will present no danger to the nation's livestock but will enable timely measures to be taken in border zones to prevent the entry of exotic virus strains. The examination of this material, under specific conditions of security, would be of immeasurable benefit, not only to Turkey but also to other countries in the Near East.

The results of a serological study of five Type A strains, isolated in Turkey, are given in Appendix 5.

## 2.4 CONCLUSIONS

One of the basic aims of the project was to increase vaccine production. To achieve this it was decided to place great emphasis on the new BHK cell suspension method for the propagation of virus. Many difficulties, both engineering and biological, were encountered and overcome so that pilot production of vaccine on an industrial scale can now begin. In addition, resources have been made available and staff trained in modern developments for vaccine production using Frenkel's method.

Many new techniques such as the automatic analysis of biological samples, the plaque forming test to measure the infectivity of FMD samples, the complement consumption test to evaluate vaccines and the separation of the virus into its important components by physical rather than chemical means have been introduced.

Solid foundations have been laid for medium- and long-term developments which will have an important impact on the control of FMD in Turkey and assist in the prevention of the spread of this major epizootic into Europe.

# Chapter 3

## RECOMMENDED FUTURE ACTIVITY

### 3.1 GENERAL PROPOSALS

Each area of operation requires intensive development. Whilst the identification of sub-types should remain the responsibility of the national authorities, it is desirable that immunological responses to vaccination of animals in the field should be correlated with the results of laboratory challenges. This procedure has already been demonstrated by the project staff but it should be amplified as it is of direct interest to the country.

### 3.2 TARGET FOR BHK CELLS IN SUSPENSION

The kinetics of the growth on BHK cells in suspension of all types and sub-types of FMD virus found in Turkey must be studied. Stocks of all strains should be established and preserved.

Present virus production on BHK cells in suspension can be improved. The major target should be a production of approximately 10 million monovalent doses per annum to be achieved as soon as possible, within a 3-year period. For this, the Government should give special attention to the optimum use of personnel. The best yield of virus from BHK cells in suspension is obtained by continuous culture. This necessitates the presence of personnel seven days a week. At present, one cell tank is used for a first cell passage each week and then a second passage is carried out in three tanks using the cells of the first tank as seed. Cell production of the second passage is always higher than that of the first. Whilst cells of the second passage are used for virus production, 20 litres are reserved at 4°C for a new cycle the following week. In other words, BHK cells are harvested once a week when they could be harvested three times weekly. This could be done using the same equipment and the same personnel working in rotation.

### 3.3 APPLICATION OF THE FRENKEL DOUBLE CULTURE METHOD

Facilities are now available to begin production on a large scale using the Frenkel double culture method. The vaccine so produced must be tested and initially guinea pigs should be used.

## 3.4 EXTRACTION AND MEASUREMENTS OF THE IMMUNOGEN COMPONENTS

Physical extraction of the immunogen component from the virus used for vaccine production has been established. The method should be used to assess the protective value of vaccines. New methods to achieve the same result, such as radial immunodiffusion, passive hemagglutination, and immunofluorescence should be investigated.

## 3.5 CORRELATION BETWEEN THE CONCENTRATION OF THE IMMUNOGEN COMPONENT AND THE RESULTS OF VACCINE CHALLENGE IN CATTLE

To ensure that the immunogen component is an accurate in vitro measuremen of vaccine potency a correlation must be established between the quantity of the immunogen component in a vaccinal dose and the degree of protection conferred in cattle.

## 3.6 METHODS TO INCREASE VACCINE EFFICIENCY

In the original Plan of Operation it was envisaged that attention would be given to the possibility of increasing the efficiency of vaccines by investigating new adjuvants, new inactivants, utilizing concentration techniques and freeze-drying. Time did not permit this work to be undertaken and these lines of enquiry should be one of the basic aims in the next three years. It is possible that the O virus, which is known to possess a low immunogenic value, may have to be concentrated before a satisfactory vaccine can be produced. In theory, either the virus or the vaccine can be concentrated. Use of modern ultra filters or precipitation by polyethylene glycol should be investigated as a means of virus concentration. Research on the action of bentonite and dextran as adjuvants and acetyl ethylenimine (AEI) and others of the same group as inactivants should be initiated.

## Appendix 1

## GROWTH OF BHK CELLS IN SUSPENSION AND PROPAGATION OF FMD VIRUS

### 1. GROWTH OF BHK CELLS IN SUSPENSION

#### 1.1 Media

Trials showed that Pirbright M6 formula gave better results than a modification of Eagle's basic tissue culture (TC) medium and it was eventually used for all TC work involving BHK cells. The original M6 medium contained pluronic polyol F68 but as supplies of this acid were difficult to obtain it was excluded. Composition of M6 medium is given in Section 2.

With the exception of mycostatin, all antibiotics used were incorporated into the medium before sterilization by filtration; mycostatin cannot be filtered and it was added to the medium as a sterile suspension, immediately before use.

#### 1.2 Equipment

The incremental production of cells required the use of containers of increasing size starting with 800 ml flasks and progressing through 5 and 10 litre New Brunswick (NB) flasks until the large-scale 150-litre tank was eventually reached.

The 800 ml flasks were equipped with a teflon rod and propeller to run at 250 rpm and included a system to permit the introduction of sterile air or carbon dioxide. The 5-and 10-litre NB flasks also possessed aeration and agitation systems; agitation was not optimal and it is probable that better cell growth could have been obtained with more efficient mixing of the medium.

The 150-litre tanks were water jacketed and equipped with an efficient spiral helix for gentle agitation; a system for surface aeration; glass electrodes which could be sterilized; and a heating system. Originally heating was by means of an element immersed in the medium; despite efficient agitation and water cooling, the element, at a high temperature in direct contact with the cells, caused considerable loss. The system was replaced by a more efficient one which ensured uniform temperature throughout the medium. Air and carbon dioxide were measured by a flow metre and passed through stainless steel filters previously sterilized with steam. The pH was automatically recorded. The glass electrodes were immersed in trypsin solution at regular intervals, between runs, to remove film of cell debris. Finally, the tanks were adapted for continuous culture of cells.

## 1.3 Multiplication of Cells

BHK cells stored at either -79°C or -190°C were thawed rapidly in a water bath at 37°C, diluted in M6 medium and 50 ml were seeded into either a Roux or a Blake flask and 100 ml into Povitsky flasks to produce monolayers. The cells adhered rapidly and the medium was changed at 24 hours. After 2 to 3 days the cellular growth from each flask was detached with trypsin/versene mixture and inoculated into three similar flasks. Experiments showed that while primary growth at two days was only sufficient to seed three similar flasks, a four day growth was usually adequate to prepare six new flasks.

Monolayer growth from two Roux or Blake or one Povitsky flask was then inoculated into 400 ml of medium in the 800 ml flasks to produce the first BHK cells in suspension.

Experimental work showed that the optimal cell density of the inoculum to produce cells in suspension was between $3 \times 10^5$ and $5 \times 10^5$ cells per ml; optimal concentration was usually achieved within 48 hours. The 48-hour growth from two 800 ml flasks was, therefore, inoculated into 4 000 ml medium in one 6-litre NB flask; each 6-litre NB flask produced sufficient cells in 48 hours to seed three 10-litre NB flasks each containing 7 000 ml medium. Finally, the growth in three 10-litre NB flasks was used to seed 100 litres of medium in the 150-litre production tanks.

In tanks used for continuous culture, 80 litres of cellular suspension was removed every 48 hours and 80 litres of fresh medium added to the 20 litres of cellular suspension remaining in the tank to restart the growth cycle.

Aeration in the culture tanks was surface only using a mixture of 95 percent air and 5 percent carbon dioxide. Trials showed that deep aeration was inhibitory to cellular growth. Approximately two to three litres of a sterile 15 percent sodium bicarbonate solution was added after 24 hours growth to maintain the pH at 7.4. The pH varied from 6.8 to 7.6 without harmful effects.

## 1.4 Harvesting BHK Cells

The classical method of harvesting large volumes of BHK cells in suspension, prior to their transfer to the virus production tank, was to allow the cells to sediment and discard the suspending medium. It had several disadvantages: the sedimentation process took 48 hours; not all the suspending medium was eliminated which meant the cells were transferred in residual medium containing 10 percent bovine serum - often containing FMD antibodies; and finally from $3 \times 10^5$ to $7 \times 10^5$ cells/ml were lost in the discarded medium.

Small volumes of cells in suspension can be separated from the growth medium by slow centrifugation using sterile precautions but there was no apparatus available capable of handling the large volumes required for commercial production of vaccine. Following pilot trials with a small centrifuge satisfactory modifications were carried out on a larger unit to enable centrifugation of substantial volumes of cells in suspension.

A centrifuge with a 40 centimetres bowl was adapted so that it could be steam sterilized and a system added to allow centrifugation in an atmosphere of filtered air under slight positive pressure. At a speed of 1 600 rpm (centrifugal force 600 g) there was no damage to the cells. Cells in growth medium were centrifuged in 100 litre quantities for 15 minutes, all the supernatant fluid was discarded and

replaced with 20 litres of M6 medium without serum; the cells were resuspended and recentrifuged; 15 litres of the washing medium was withdrawn and discarded; the centrifuge was started once more and then stopped using a brake. The braking caused the deposited cells to become resuspended in the remaining 5 litres of washing medium, forming a perfect cell suspension containing approximately 6 x $10^7$ cells/ml. The dense suspension was removed by vacuum and added to the virus production tank; 300 litres of cells in suspension were handled per hour.

Harvesting cells by centrifugation greatly increased the virus production potential of the available equipment and plant.

## 1.5 Virus Propagation

Growth of FMD virus strains on BHK cell monolayers did not present any problems. Virus was harvested when the majority of cells were detached, as the infectivity titre was highest at this time. The virus had to be passaged 6 or 7 times in BHK cells in suspension before it became completely adapted to the new form of growth. Once adaptation was complete, strains were preserved as seed banks at $-79^{\circ}$ or $-190^{\circ}$C. Adapted strains were inoculated into virus production tanks of 150-litre capacity containing 100 litres of cellular suspension in medium M6 without serum, penicillin or streptomycin but with additional sodium bicarbonate. The tanks were double-jacketed and equipped with aeration, pH monitoring, heating and agitation systems.

Trials were carried out with BHK cell suspensions which varied in density from 1.5 x $10^6$ to 4 x $10^6$ cells/ml to find the optimal cell density for virus production. A density of 2.5 x $10^6$ cells/ml was finally chosen for large-scale virus growth.

Infected BHK cells dying from the 24th to 30th hour usually showed a peak of infectivity at 16 to 18 hours. It was determined that from 1.0 to 2.0 percent of a culture, at its peak of infectivity, was required to initiate satisfactory replication in the virus production tank.

It was found essential, during virus replication, to maintain a constant pH of 7.2. Trials showed that if the pH rose to 7.6 only 60 percent of the cells were destroyed compared with 80 percent at pH 7.2; (destruction of cells is a good yardstick for the measurement of virus production). Further work showed that once a virus was adapted to BHK cells in suspension, up to 95 percent of the cells were destroyed in 24 hours. The pH of the medium was maintained at 7.2 by increasing the initial content of sodium bicarbonate in the 6M formula and by adding four to six litres of sterile 15 percent sodium bicarbonate solution to the production tank as necessary during virus growth.

It has long been recognized that O strains of FMD virus are, in comparison with A strains, poor immunizing agents. This was confirmed by the fact that whereas 2.0 ml virus vaccine Type A ($A_{22}$ and $A_{28}$) protected all cattle exposed to challenge, 4.65 ml of Type O vaccine were required to give similar protection. Working with 3.6 ml Type O vaccines produced from a single tank and harvested at 18, 24, 30 and 42 hours indicated that the virus should be harvested at 24 hours and this period of incubation was adopted. At this time destruction of cells is virtually complete, infectious titre is still high and antigenic titre is at the level of the plateau.

## 2. COMPOSITION OF M6 MEDIUM

### AMINO ACID CONCENTRATE FOR M6 MEDIUM

| | |
|---|---|
| L – Arginine | 8.4 g |
| L – Cystine (dissolve in 10 ml Nl NaOH) | 4.8 g |
| L – Histidine monohydrochloride | 3.84 g |
| L – Iso–Leucine | 10.48 g |
| L – Leucine | 10.48 g |
| L – Lysine monohydrochloride | 14.62 g |
| L – Methionine | 3.0 g |
| L – Phenylalanine | 6.6 g |
| L – Threonine | 9.52 g |
| L – Tryptophan | 1.6 g |
| L – Tyrosine | 7.24 g |
| L – Valine | 9.36 g |
| Inositol | 0.7 g |
| Phenol red (sol 0.1%) | 4.0 ml |
| Distilled water up to | 10 litres |

Dissolve L–cystine separately in 10 ml normal NaOH; divide the remaining amino acids into two parts; add each part to a glass or stainless steel container with four litres of distilled water; heat to 60°C for 20 to 30 minutes; pool both halves; add the cystine and phenol red.

Check pH on metre, neutralize to pH 7.0, using N/1 HCl or N/1 NaOH. Make volume up to 10 litres with distilled water. Divide and store at -20°C.

## VITAMIN CONCENTRATE FOR 6M MEDIUM

| | |
|---|---|
| Choline chloride | 0.5 g |
| Folic acid (dissolve in 50 ml N/1 NaOH) | 0.5 g |
| Nicotinamide | 0.5 g |
| DL - Pantothenic acid | 0.5 g |
| Pyridoxaline hydrochloride | 0.5 g |
| Thiamin hydrochloride | 0.5 g |
| Riboflavin | 0.05 g |
| Distilled water up to | 1 litre |

Dissolve separately folic acid in 50 ml of N/1 NaOH;

Dissolve the remaining vitamins in 700 ml distilled water;

Add the folic acid solution and make up volume to 1 litre with distilled water.

Divide and store at -20°C.

## TRYPTOSE LACTALBUMIN CONCENTRATE FOR 6M MEDIUM

| | |
|---|---|
| Tryptose phosphate broth powder | 29.5 g |
| (or trypticase soy broth) | (30.0 g) |
| Lactalbumin hydrolysate | 25 g |
| Distilled water | 1 litre |

Dissolve in glass or stainless steel container at 80°C until solution is perfectly clear; sterilize at 121°C for 15 minutes.

## ANTIFOAM FOR 100 LITRES OF M6 MEDIUM

| | | |
|---|---|---|
| - | Antifoam Emulsion RD[1/] | 1 ml (up to 40) |
| - | Distilled water | 100 ml (up to 100) |

Sterilize by autoclaving at 121°C for 30 minutes.

## PHENOL RED SOLUTION 0.1% FOR M6 MEDIUM

| | | |
|---|---|---|
| - | Phenol red | 1 g |
| - | NaOH (0.1 N) | 29.2 ml |

Grind phenol red with the one tenth normal NaOH in pestle and mortar; make up to one litre with distilled water; filter through ordinary filter paper.

## ANTIBIOTICS FOR M6 MEDIUM

Penicillin Solution

- Use 100 units per ml
- Each vial generally contains 100 000 units
- Dissolve contents of 1 vial in 10 ml of sterile distilled water
- Use 1 ml of the solution for 1 litre medium.

---

1/ Midland silicons.

Streptomycin Solution

Idem to penicillin.

Bacitracin Solution

- Titre is given for the batch, for instance T=64 units/mg
- 2 500 units are used for 1 litre of culture medium and are dissolved in 1 ml phosphate buffer pH 6.0
- 2 500 units correspond to $\frac{2\ 500}{64}$ = 39 mg
- Preparation of the solution

  (a) Bacitracin powder $\frac{39 \times 100}{1\ 000}$ = 4 gr (approx.)

  (b) Buffer pH = 6.0 100 ml

- Filter through EKS; store stock solution at -20°C.
- Bacitracin solution is used at the ratio of 1 ml for 1 litre of medium; store solution ready for use in the refrigerator.

Colimycin Solution

- Titre is given for the batch, for instance T = 19 500 units/mg
- 200 000 units are used for 1 litre of culture medium and are dissolved in 1 ml phosphate buffer pH = 6.0
- 200 000 units correspond to $\frac{200\ 000}{19\ 500}$ = 10.256 mg
- preparation of the solution:

  (a) colimycin powder $\frac{10.25 \times 100}{1\ 000}$ = 1.025 g

  (b) buffer pH = 6.0 100 ml

  Filter through EKS, store stock solution at -20°C.

  Colimycin solution is used at the ratio of 1 ml for 1 litre medium; store ready use solution in the refrigerator.

Neomycin (base) Solution

- Use at the ratio of 100 mg per litre
- Dissolve 100 mg of neomycin in 1 ml of buffer solution at pH 8
- Preparation of the solution

  (a) neomycin powder $\frac{100 \times 100}{1\ 000}$ = 10 g

  (b) buffer pH 8.0 = 100 ml

  Filter through EKS; store stock solution at -20°C.

  Neomycin solution is used at the ratio of 1 ml for 1 litre of medium; store ready use solution in the refrigerator.

Mycostatin Suspension

- Each vial contains 500 000 units
- For 1 litre of medium use 50 000 units which is suspended in 1 ml of sterile distilled water
- Preparation of the suspension

  Vial 1

  Sterile distilled water 10 ml

  To be prepared when required, store surplus in the refrigerator.

Buffer Solution at pH = 6

| | |
|---|---|
| $K_2HPO_4$ | 2 g |
| $KH_2PO_4$ | 8 g |
| Distilled water | 1 000 ml |

Buffer Solution at pH = 8

| | |
|---|---|
| $K_2HOP_4$ | 16.73 g |
| $KH_2PO_4$ | 0.523 g |
| Distilled water | 1 000 ml |

## MEDIUM FOR CELL STORAGE AT -79 or -190°C

| | |
|---|---|
| 6M medium | 80% |
| Glycerine | 10% |
| Serum | 10% |
| Antibiotics | |

## M6 MEDIUM FOR BHK CELL CULTURE AND VIRUS GROWTH

| | | | |
|---|---|---|---|
| Distilled water | | 80 | l |
| Ferric nitrate, sol. 0.1% Fe $(NO_3)_3$ $9H_2$ | | 8 | ml |
| Phenol red, sol. 0.1% | | 800 | ml |
| Vitamin concentrate | | 640 | ml |
| Amino acid concentrate, heated at 60°C | | 1 000 | ml |
| Salts : | NaCl | 512 | g |
| | KCl | 32.1 | g |
| | $MgSO_4$ $(7H_2O)$ | 16 | g |
| | Glucose | 620 | g |
| | $NaH_2PO_4$ $(2H_2O)$ | 11.20 | g |
| | L - Glutamine | 46.60 | g |
| | $CaCl_2$ | 16 | g |
| | $NaHCO_3$ | 220 | g |
| Antibiotics: | | | |
| | Bacitracin powder | 5.5 | g |
| | Colimycin powder | 2.0 | g |
| | Neomycin powder | 10.0 | g |
| Tryptose, lactalbumin, heated at 80°C | | 10 | l |
| Clarified bovine serum or distilled water for virus culture | | 10 | l |

pH adjustment : Bubble through with $CO_2$ until pH 7.2

Filtration through Seitz 40 x 40 7 sheets EKS

## Appendix 2

## TITRATION OF ACTIVITY (PFU TEST) AND DETECTION OF FMD VIRUS USING THE PLAQUE METHOD

### CELLS

Three cell lines, susceptible to FMD virus, are maintained; each has advantages and disadvantages. They are:

(i) RBHK, a BHK 21 line from the Razi Institute, Teheran, Iran;

(ii) PBHK, also a BHK 21 lines, clone 13, from the Virus Diseases Research Institute, Pirbright, England;

(iii) BA, a porcine kidney line from the Teheran branch of IFFA located at the Razi Institute.

The RBHK grows well in Hank's medium containing bovine serum and yeast extract; the growth medium for the remaining two lines is modified Eagle's also with bovine serum and yeast extract.

PBHK and BA cells are about 10 times more susceptible to Types O and A virus than RBHK cells. Due to their high susceptibility, they are suitable for the detection of small amounts of virus, for example, in poor field samples. They may also be useful in tests for complete inactivation of virus in cultures intended for vaccine production, when formalin is replaced by more modern inactivants.

Despite lower susceptibility, the RBHK line was chosen for plaque tests because it is easier to handle; it adheres well on glass and remains alive in the refrigerator for more than one month. In contrast, PBHK cells have a tendency to detach from the glass and they do not remain alive as long in the refrigerator, 3 weeks at the most. The BA line grows more slowly and it is more difficult to predict when the cell layers will be complete. This is a considerable disadvantage for the programming of plaque tests. Cultures of BA cells die off rapidly in the refrigerator; they are kept at room temperature, after incubation, but do not remain alive for more than 11 days. A further disadvantage is that monolayers of these cells do not show as smooth edges as RBHK cells, and so it is more difficult to recognize marginal plaques with the naked eye. Microscopic examination is required more often than with RBHK cells.

### PREPARATION OF CULTURES FOR PLAQUE TESTS

Experience has shown that cultures in 110 x 150 mm test tubes are well suited for plaque tests; they do not require a $CO_2$ atmosphere and take much less incubator space than Petri dishes or bottles; plaques can be readily counted using the microscope for very small or doubtful marginal plaques. The amount of growth medium per tube is 2 ml.

Dense monolayers are obtained after stationary incubation at 37°C for 24 to 72 hours depending on the density of the cell seed. They are washed with 2 ml of VM 3 (Appendix 2A), per tube, to remove the antibody-containing growth medium. All dispensing is done with Cornwall syringes.

## VIRUS TITRATION

Virus samples to be titrated are diluted serially in VM 3, the dilution factor depending on the degree of accuracy required (the smaller the factor, the higher the precision). In routine titrations of culture samples, 10-fold serial dilutions are made using a fresh sterile pipette for every dilution. The dilutions, starting with the highest one, are pipetted in 0.2 ml amounts onto the washed monolayers using 4 tubes per decimal dilution. For virus adsorption, the tubes are kept at 37°C for 60 minutes, tilting the culture racks gently every 15 minutes to achieve even spreading of the inoculated material over the cell monolayers.

After the adsorption period, the seed material is not removed; each cell is covered with 2 ml Tylose-VM 3 overlay medium (Appendix 2A) to prevent further spread of the virus. The overlay medium is well mixed with the serum-virus mixture already in the tubes by rotating the tubes gently in a near-horizontal position; they are then incubated at 37°C for 42 hours. Care must be taken during this period to avoid shaking the incubator or culture racks as even the slightest vibration or motion may distort the plaques under the viscous overlay. Plaques are normally round and distorted plaques show tails and other irregularities.

After incubation, the cell sheets are stained with Giemsa's stain as described in Appendix 2A; plaques are counted visually using a titration illuminator and an inverted microscope when necessary, especially in the case of very small or doubtful marginal plaques. The size of the plaques caused by FMD virus varies considerably; strains making large and very small plaques occur within the same virus type. For titrations, virus strains making large or medium size plaques are preferable.

## CALCULATION OF INFECTIVITY TITRES

Plaque titres are calculated according to a formula based on the Poisson distribution and introduced in Ankara by D. Stellman, IFFA, Lyon. The following is an example from a routine titration of BHK suspension-culture virus:

| Dil. | Dil. factor (f) | No. of tubes (n) | f x n | No. of plaques in individual tubes | Total No. of plaques |
|---|---|---|---|---|---|
| $10^{-3}$ | | | | not countable | |
| $10^{-4}$ | 10 | 4 | 40 | 15 18 22 19 | 74 |
| $10^{-5}$ | 1 | 4 | 4 | 2 3 0 3 | 8 |

$$f \times n = 44$$

$$m = \frac{N}{f \times n} = \frac{82}{44} = 1.86 \times 10^5 \text{ PFU/0.2 ml} = 10^{5.27} \text{ PFU/0.2} = 10^{5.97} \text{ PFU/1 ml}$$

Precision: $\frac{2}{\sqrt{N}} = \frac{2}{\sqrt{82}} = \frac{2}{9.06} = 0.22$

$$\log\left(1 + \frac{2}{\sqrt{N}}\right) = \log 1.22 = 0.09$$

Calculated infectivity titre: $10^{5.97} \pm 0.09$ PFU/ml

The results of plaque titrations are more precise when a smaller dilution factor, e.g. 4, is used or when the number of cultures per virus dilution is increased; in very precise titrations, four-fold serial virus dilutions should be made and the number of cultures per dilution increased to eight.

## PLAQUE-REDUCTION (NEUTRALIZATION) TEST

Small amounts of neutralizing antibody can be detected in animal sera using the plaque-reduction (neutralization) test. Sera may be tested without inactivation or after heating to 58°C for 30 minutes.

The serum is filtered through a Sartorius membrane filter mounted on a 20 ml Record syringe and two-fold serial dilutions are mixed with a virus dilution containing 100 to 200 PFU/ml as shown by a previous infectivity titration; VM 3 is used as diluent for both virus and serum. The mixtures of serum and virus are kept in a waterbath for 30 minutes at 37°C. Dense, test-tube cultures of RBHK cells, 4 to 8 per preparation depending on the precision required, are used to test the mixtures for infectivity; the cell sheets are washed with VM 3, 2 ml per tube; and 2 ml of the test preparations are added to each tube. In every test, a known positive and a normal serum originating from the same animal species as the serum under test are included as controls and other control tubes contain VM 3 istead of serum. The adsorption period, the incubation time following addition of Tylose-VM 3 overlay medium, and the staining procedure are the same as described above for the infectivity titration.

The total number of plaques present in all tubes of every serum dilution is compared with the sum of the plaques present in the same number of control tubes containing VM 3 instead of serum. For every serum, the highest dilution reducing the plaque count by 50 percent compared with the VM 3 control (100 percent) is determined graphically, plotting the percentages of plaques against the $\log_{10}$ of the serum dilutions on millimetric graph paper.

With the serum-dilution method, which is theoretically the best, it is often difficult to judge the proper virus dilution to produce the optimal number of plaques for easy counting in the VM 3 controls. Deep-frozen samples of the same infectious culture fluid may vary considerably in titre when tested on different days and in different batches of the same cells. This difficulty can be overcome by carrying out checkerboard tests in which serial (two-fold) serum dilutions are tested against serial (4-fold or 10-fold) virus dilutions. This procedure is too laborious for routine tests.

Since many normal sera contain non-specific inhibitors reacting with FMD antigen at lower serum dilutions, it is necessary to determine the limit between neutralization caused by serum inhibitor and that due to specific antibody using a large number of sera from animals of known susceptibility. This limit has not yet been established for Turkish cattle. It can only be stated that normal bovine serum purchased in the U.S.A., where FMD does not exist, sometimes reduced the plaque count by 50 percent in a dilution of 1:4. This serum was used as the negative control in the plaque-reduction tests. It is quite likely, however, that in some cattle a higher serum dilution than 1:4 represents the limit between non-specific and specific reactions.

VIRUS-DILUTION METHOD

The difficulty of selecting the most favourable virus dilution does not exist when the virus-dilution method is used. In this case, a constant amount of serum is tested against serial virus dilutions. In view of the mode of reaction of American normal serum, an arbitrary serum dilution of 1:8 was chosen for routine tests using this method. This, however, involved the risk that very small amounts of antibody might not be detected even though the test is very sensitive.

In plaque-reduction tests using the virus-dilution method virus is titrated in presence of:

(a) serum under test
(b) positive control serum
(c) negative control serum
(d) VM 3

} diluted 1:8 in VM 3.

The dilution range of the test virus depends on the result of a preceding infectivity titration (plaque method) in presence of VM 3. In RBHK cells, plaque titres of relatively potent samples of virus cultures lie in the neighbourhood of $10^6$ PFU/ml. Consequently, if 10 is used as dilution factor, a virus dilution range of $10^{-1}$ to $10^{-6}$ should be selected for the test and positive control sera and a range of $10^{-3}$ to $10^{-6}$ for the normal control serum and VM 3. If the dilution factor is 4, the virus dilution ranges would be $4^{-1}$ to $4^{-10}$ for test positive serum and $4^{-5}$ to $4^{-10}$ for the negative serum and the VM 3 control. The number of tube cultures to be used per mixture depends on the precision desired; at least 6 tubes should be used when the dilution factor for the virus is 10 and at least 4 tubes when sera are tested against 4-fold virus dilutions.

Equal quantities of the serial virus dilutions in cold VM 3 and of the 1:8 serum dilutions are mixed and the preparations incubated in a waterbath for 30 minutes at 37°C; 0.2 ml amounts are then pipetted onto washed monolayers of RBHK cells. Note now that the amount of virus dilution per tube is 0.1 ml, which must be considered in the calculation of the neutralization index. The adsorption period, the incubation time after addition of Tylose-VM 3 overlay medium and the staining procedure are the same as described for the infectivity titration.

The neutralization index of a serum is obtained by subtracting the -log of the infectivity titre of the virus in the presence of the 1:8 serum from the $-\log_{10}$ of the titre in the presence of VM 3. These titres are computed according to the formula given above in the section on infectivity titration. Since the dose of the virus, per tube, in the plaque-reduction test is only half of that in the $\log_{10}$ (1) must be added to the log of the number of PFU/0.1 ml instead of $\log_{5}$ (0.7).

In plaque-reduction tests made with sera from 25 cattle originating from the Antalya district, where vaccination is not practised and the cattle are susceptible, the highest neutralization index found was 0.43 log for $O_1$ and 0.07 log for $A_{22}$ viruses. For some unknown reason, the indices lay generally lower with Type A than with Type O viruses; it is considered that indices higher than 0.5 log are suggestive of specific neutralization.

## Appendix 3

# SEPARATION BY ZONAL ULTRA CENTRIFUGATION OF COMPONENTS OF FMD VIRUS AND DETERMINATION OF THEIR TITRE BY COMPLEMENT FIXATION

Zonal ultra centrifugation is tending to replace flurocarbon extraction as a means of determining the immunogenic value of foot-and-mouth disease vaccines 1/ 2/. The vaccines are known to possess two important components: the large particles of 22 nm 1/ and the small particles of 7 nm with a sedimentation constant of 140W and 14S respectively. Both particles have the ability to fix complement but only those with a sedimentation constant of 140S possess the power to immunize 3/.

Zonal ultra centrifugation presents a method of separating out the large particles from a vaccine and quantitative measurements can be made using the complement fixation (CF) test.

A standard method has been developed to separate out the nm particles, using a saccharose gradient, and to carry out quantitative measurements.

## MATERIAL AND METHODS

The CF titre of a Frankel O-type vaccine was determined; 1 000 ml of the vaccine was concentrated to 100 ml in a rotary evaporator; 50 ml aliquots were centrifuged at 5 000 rpm for 20 minutes and the supernatant fluid retained as concentrated antigen for test.

Saccharose solutions of 15, 45 and 50 percent (weight/weight) were prepared in Mayer and Levines' buffer solution.

Centrifugation was carried out in a zonal ultra centrifuge equipped with a B14 rotor having a capacity of 650 ml; accessories for continuous flow were connected to ancillary equipment which included two cylindrical reservoirs, a vibrator and a peristaltic pump of variable speed shown schematically in Figure 1.

The centrifuge was set to run at between 2 500 and 3 000 rpm; 400 g of 15 and 45 percent saccharose solutions were placed in reservoirs B and A respectively, they reached equilibrium through a connecting tube when it was opened; the vibrator and peristaltic pump were started and the gradients forced into the rotor. The lighter gradient occupied the periphery of the rotor but as more gradient was forced in this was gradually replaced by the denser solution, the lighter gradient finding its way to the centre of the rotor. When approximately 600 ml gradient had been introduced, at a rate of 20 ml per minute, the flow was stopped; 50 percent gradient was then introduced directly into the rotor through tube C; when the lightest gradient began to flow from tube D the rotor was completely filled with saccharose gradient which was stabilized by centrifugal force and increased in density in a linear manner from 15 percent at the centre of the rotor to 50 percent at the periphery.

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1/ Nanometre is used in place of millimicron to comply with current practice.

The pump was transferred to tube D and reservoirs B and A were disconnected; 50 ml concentrate antigen was pumped in via tube D displacing an equal volume of 50 percent gradient which flowed out by tube C; tubes C and D were closed.

Plate E was removed and the rotor cowl was placed in position; the centrifuge was closed and speed increased to 43 000 rpm; at a temperature of 10°C. After three hours centrifugation speed was reduced to 3 000, the cowl was removed and plate E replaced. The pump, having been washed with 50 percent gradient solution, was reconnected to tube C and started. Gradient solution containing the respective virus components was collected via tube E, the lightest fractions first; in all 13 different fractions were collected.

The fractions were diluted 1:2 with Mayer and Levines' buffer and their titre determined in an autoanalyzer.

RESULTS

Very sharp separation of the 7 and 22 nm particles was obtained and it is evident that the ease with which separation was effected was due to the marked difference in their constants of sedimentation.

Figure 2 shows that fractions 1, 2 and 3 contained 7 nm particles, fractions 10 to 13 inclusive 22 nm particles and that fractions 4 to 9 inclusive had minimal or no detectable activity.

The work was repeated several times giving comparable results; the peaks were not always of the same magnitude but this was a function of individual antigens.

Fifty millilitres of concentrated antigen was used and 13 different fractions tested; each fraction represents one thirteenth of the total antigen content of the original vaccine and the sum of the titres should be equal, or nearly equal to the titre of that vaccine. Thus, to prepare Figure 2 the following data were available.

Titre of fractions multiplied by two to take into account primary dilution factor.

| | | |
|---|---|---|
| Fraction 1 | Titre = 26 x 2 = 52 | Titre of the 7 nm particles was 168 |
| Fraction 2 | Titre = 32 x 2 = 64 | |
| Fraction 3 | Titre = 26 x 2 = 52 | |
| Fraction 10 | Titre = 22 x 2 = 44 | Titre of the 22 nm particles was 240 |
| Fraction 11 | Titre = 32 x 2 = 64 | |
| Fraction 12 | Titre = 41 x 2 = 82 | |
| Fraction 13 | Titre = 25 x 2 = 50 | |

The total of the titres for the fractions was 408 which may be compared with a titre of 420 for the original vaccine. Thus practically all the initial antigenicity was accounted for.

## CONCLUSION

Generally speaking ultra centrifugation using a zonal rotor type B offers several advantages over the previous method using centrifuge tubes. 50 to 60 ml of antigen can be utilized instead of 2.0 ml and it is thus possible to obtain greatly increased quantities of the antigenic fractions for experimental work. Preparation of gradients, ultra centrifugation, and collection of the fractions is a continuous process and the stabilization of the gradients is ensured by centrifugal force.

This automated process leads to the preparation of specimens in a way which can be reproduced continuously with great precision.

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1/ M.T. Fayet, M.Roumiantzeff and D.Fargeaud: Rev. Immunol., 33, 1969, 335-344.

2/ G.N. Mowat: Personal Communication, 1970.

3/ F.Brown and J. Crick: J.Immunol., 82, 1959, 444.

Figure 1

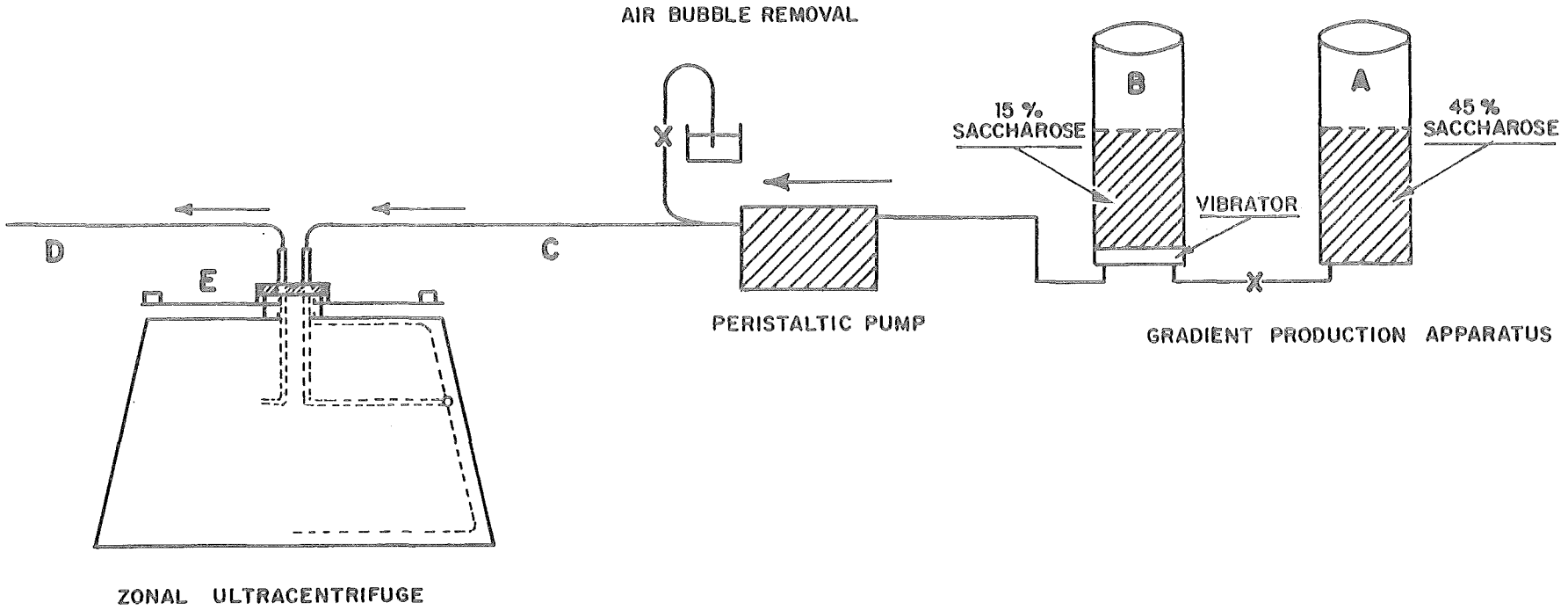
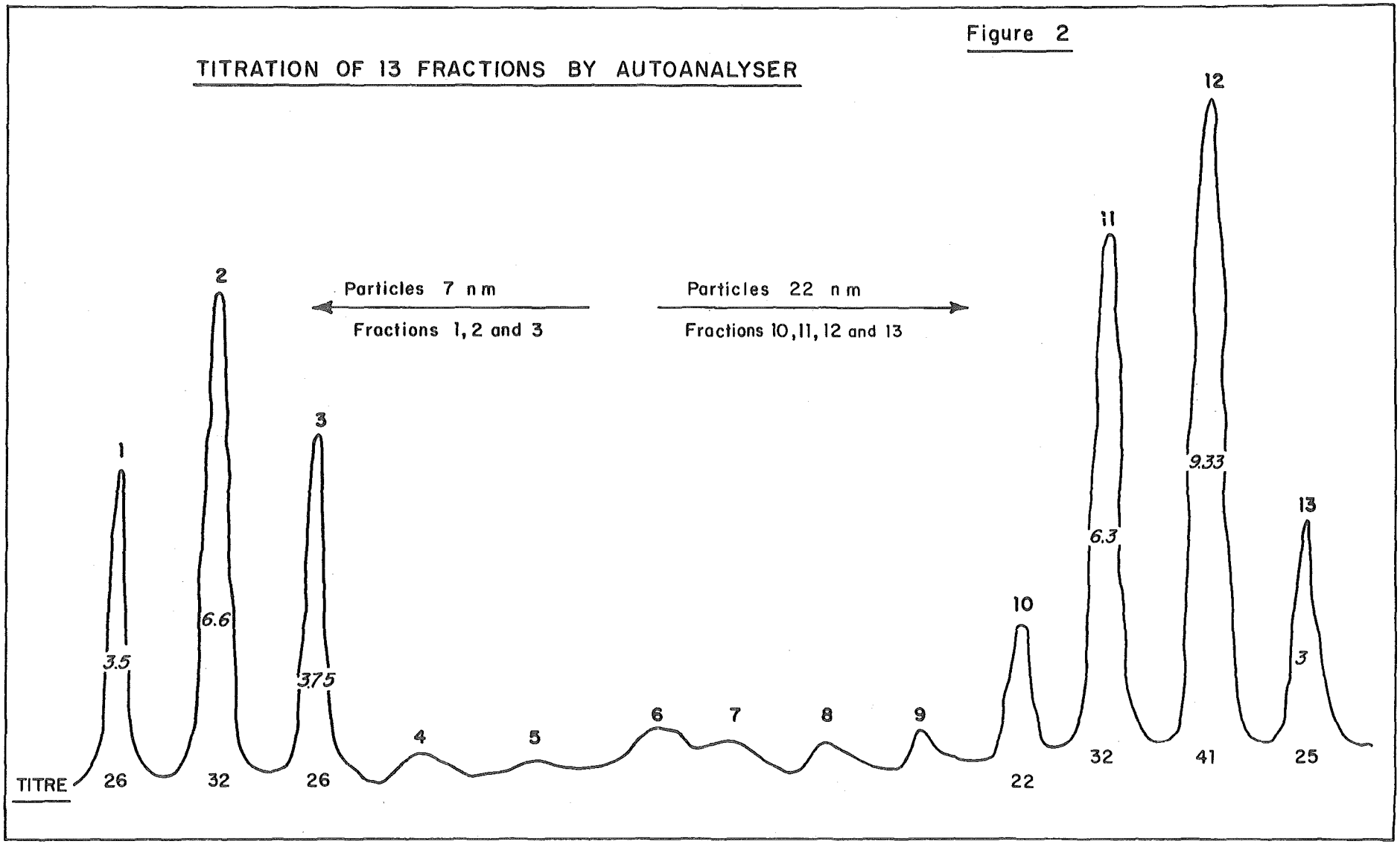

AIR BUBBLE REMOVAL
15 % SACCHAROSE
B
A
45 % SACCHAROSE
VIBRATOR
D
E
C
PERISTALTIC PUMP
GRADIENT PRODUCTION APPARATUS
ZONAL ULTRACENTRIFUGE

Figure 2

TITRATION OF 13 FRACTIONS BY AUTOANALYSER



## Appendix 4

# AN INDIRECT METHOD OF TESTING THE POTENCY OF FMD VACCINES IN ADULT MICE

## (IMPROVED TECHNIQUE)

As FMD virus is usually not pathogenic in adult mice, an indirect method was developed 1/ 2/ by which the antibody response in mice to FMD vaccine is tested by direct complement fixation (CF) using cold fixation. The following detailed description is given of the improved technique now in use, including some results obtained in comparative vaccine tests in mice and cattle.

## VACCINATION AND SERUM COLLECTION

Adult mice (32 per monovalent vaccine) receive 0.5 ml undiluted vaccine subcutaneously in one flank, the needle being inserted from above so that there is no backflow of inoculated material. If the vaccine does not contain too much saponin, that is, more than 0.3 mg/ml, the animals show no reaction except some swelling at the site of inoculation. Higher saponin concentrations are toxic and may decrease antibody (Ab) production by the animals. Sera from mice severely intoxicated by saponin may be strongly anticomplementary. Three weeks later the animals are bled from the heart, taking 0.3 ml blood from each mouse; not much pressure should be exerted in expressing the blood from the syringe into the centrifuge tube since mouse erythrocytes rupture easily releasing hemoglobin into the serum. A blood pool is made from all mice in the test and the serum separated by centrifugation; it is inactivated at 58°C for 30 minutes.

Since serum titres of individual mice may vary greatly, it is necessary to make serum pools from large batches of mice. Calculations have shown that 32 animals per monovalent vaccine are sufficient.

## ANTIGENS FOR CF TESTS

The antigens to be used depend on the composition of the vaccine. The virus type corresponds to that of the vaccine. Vaccinated mice will develop Ab not only against the viral antigen (Ag) but also against other antigenic substances contained in the vaccine. The mass of this non-specific material is much greater than that of specific Ag. When cold fixation is practised, which intensifies CF considerably, the non-specific Ab may disturb the reaction so much that conclusive results are not obtained.

In Turkey, Frenkel-type and BHK vaccines are produced. Quite often vaccines of both kinds are mixed. In this case it is sometimes difficult to get clear-cut results in CF tests with sera from vaccinated mice. It is fortunate in this respect that the vaccine virus is routinely treated by chloroform, which evidently precipitates some of the non-specific Ag. Vaccines made from BHK virus treated with chloroform do not often elicit a non-specific Ab response strong enough to make the results of CF tests inconclusive.

It is now the general rule to use Ag, prepared from BHK cells, against sera from mice inoculated with Frenkel vaccine and Ag made from Frenkel-culture virus for sera from mice vaccinated with BHK vaccine. However, either Ag may cause difficulties with serum from mice which received a mixture of both kinds of vaccine.

BHK antigen is prepared from infected RBHK cells (see Appendix 2) grown in 3-litre rolling bottles using 100 ml Hanks' medium per bottle and a very dense cell seed. When the cell layers are complete, they are washed with VM 3 (50 ml per bottle) to dilute out Ab present in the growth medium. The washing fluid is decanted and replaced by 50 ml fresh VM 3 containing 1 ml infectious culture fluid. The bottles are then returned to the roller and incubated until the infected cells have detached from the glass, usually 24 hours. Since much Ag remains in the rounded detached cells, the culture fluid containing these cells is centrifuged at 2 500 rpm for 20 minutes. The supernatant fluid is not used but the cell sediment is weighed and resuspended in 1/10 of the original amount of supernatant fluid and frozen overnight or longer at -20 to -25°C; after thawing, the cell suspension is cleared by centrifugation and the supernatant represents the Ag; its CF titre is usually greater than 1:8.

Due to the fact that inactivated pools of mouse serum often show some anti-complementary activity, CF tests with murine sera require Ag showing no anti-complementary effect when tested in presence of decreasing amounts of complement (C') in comparison with buffered saline. To ensure freedom from anticomplementary activity, Ag is shaken mechanically with chloroform (1/10 vol) for 60 minutes in the cold room and thereafter the precipitate is removed by centrifugation.

Normal BHK antigen required as a control in CF tests is prepared from monolayers of non-infected RBHK cells grown in 1-litre Blake bottles or in larger Povitzky bottles. The cells are removed from the glass with a metal scraper made from strong wire; suspended in maintenance medium (VM 3) and centrifuged; the sediment is weighed and resuspended in a calculated quantity of supernatant fluid to give the same wt/vol ratio as in the infectious Ag; the suspension is frozen, thawed and cleared by centrifugation as described above. Normal Ag is tested for anticomplementary effect in the same way as infectious Ag and treated with chloroform if necessary.

Frenkel antigen, clarified extract of infected cattle tongue epithelium, is concentrated five to seven times by vacuum evaporation and dialyzed against physiological saline for 24 hours, changing the saline once, to remove salts present in high concentration as these have a strong anticomplementary effect. Dialysis is carried out, with magnetic stirring, in the cold room; since the volume of the material increases in an unpredictable way during dialysis it is preferable to concentrate it about eight to ten fold to achieve a final concentration of five to seven fold.

As a control, an extract is prepared in the same way from non-infected cattle tongue epithelium; it is concentrated in the same manner as the infectious Ag; if it is anticomplementary, it is treated with chloroform.

## REAGENTS FOR CF TEST

(1) Mouse serum pool under test, inactivated at 58°C for 30 minutes (MS);

(2) positive reference serum from mice vaccinated with the same type of vaccine and known to have immunized cattle;

(3) inactivated normal mouse serum (MNS);

(4) infectious Ag (Frenkel Ag concentrated in Rotavapor);

(5) normal Frenkel Ag concentrated by vacuum evaporation, strictly comparable with No.4;

(6) preserved C';

(7) hemolytic system with 3 units of hemolysin (HS).

## QUANTITIES OF REAGENTS, FIXATION AND LYSIS PERIODS

| | | | |
|---|---|---|---|
| Murine serum, undil. or in dil. | 0.05 ml | 21 hours | |
| Ag, undil. or in dil. | 0.05 ml | at + 2°C | 60 minutes |
| C' dilution | 0.1 ml | | |
| Phosphate-buffered saline | 0.1 ml | | at 37°C |
| HS | 0.2 ml | | |
| Total: | 0.5 ml | per tube | |

## C' TITRATION

C' preserved with Witte's fluid is used. It is kept deep-frozen in test tubes and thawed as needed. Thawed C' not immediately used may be kept in an ice bath (to be renewed daily) in the refrigerator at 4°C; under such conditions preserved C' retains activity for several weeks.

Before setting up the CF test, C' is titrated in presence of the following reagents using 0.1 ml pipettes for 0.05 and 0.1 ml amounts:

| | | | | | | |
|---|---|---|---|---|---|---|
| (1) | MS under test | (0.05 ml) | + | buffered saline | (0.05 ml) | all reagents undiluted: 8 tubes in each row |
| (2) | reference MS | " | + | " | " | |
| (3) | MNS | " | + | " | " | |
| (4) | infectious Ag | " | + | " | " | |
| (5) | normal Ag | " | + | " | " | |
| (6) | MS under test | " | + | normal Ag | " | |
| (7) | reference MS | " | + | " | " | |
| (8) | MNS | " | + | " | " | |
| (9) | MNS | " | + | infectious Ag | " | |
| (10) | buffered saline (0.1 ml) | | | | | |

To all tubes are then added 0.1 ml buffered saline and 0.1 ml C' dilution (1 tube in each row for each C' dilution) ranging from three to ten percent. C' is previously diluted separately (for instance: 3% C' = 0.6 ml 10% C' + 1.4 ml buffered saline; 4% C' = 0.8 ml 10% C' + 1.2 ml saline; etc.).

The tubes are sealed with 000 rubber stoppers and refrigerated, preferably at 2°C for 21 hours. Thereafter 0.2 ml HS are added to each tube followed by incubation in a 37°C waterbath for 60 minutes. The percentage of unlysed cells in each tube is estimated visually using the following marking system: 4 + = 100% (no hemolysis); 4 + = 94%; 3.5 = 87%; 3 + = 75%; 2.5 + = 62%; 2 + = 50%; 1.5 + = 37%; 1 + = 25%; 0.5 + = 12%; tr (= trace) = 6%; 0 = complete hemolysis. In the CF test, the strength of C' to be used is increased by one percent compared with its minimal lysing dose in the respective titration row. If, for instance, 7% C' lysed all cells in row 6 containing MS under test + normal Ag, 8% C' is used for this combination in the CF test. A combination with infectious Ag is only possible for MNS because Ab-containing sera will fix C' in presence of infectious Ag and thus give erroneous results in the C' titration. Since Ag are usually less anticomplementary than MS, one percent more C' is used for combinations of MS expected to contain Ab plus infectious Ag than the amount required for complete hemolysis in presence of the MS alone.

CF TEST

This test is now set up in checkerboard form, that is, undiluted MS and serial dilutions of from 1:2 to 1:16 are tested against undiluted Ag and serial dilutions of from 1:2 to 1:16; MNS is used undiluted only in presence of undiluted Ag and Ag dilutions 1:2 and 1:4; every test contains control tubes in which either MS (in Ag controls) or Ag (in serum controls) is replaced by buffered saline. Since murine sera are sometimes hemolytic, the serum controls without C' (SC 2) are important for the colour comparison of the supernatants after sedimentation of the unlysed cells either by centrifugation or after standing overnight in the refrigerator.

The form and results of a checkerboard test are given in Table 1. It shows that the controls were correct; that the serum under test contained Ab; and that the zone of Ag-Ab equivalence, in which serum titres are calculated, was not the same for both positive sera. The MS under test reacted best with an Ag dilution of 1:2 whereas the reference serum reacted most strongly with an Ag dilution of 1:4.

In these zones of equivalence, the serum dilutions allowing 50 percent hemolysis are determined on millimetric graph paper, plotting the estimated percentages of unlysed cells against the $\log_{10}$ of the serum dilutions. In this way a serum titre of 1:4 is obtained for the serum under test and a titre of 1:7.5 for the reference serum. This indicates that the vaccine under test had a lower immunizing power in mice than the reference vaccine.

## COMPARISON OF RESULTS OF CF TESTS OF SERA FROM VACCINATED MICE WITH THOSE OF POTENCY TESTS IN CATTLE

The first experiment (Fig. 1) was carried out in Germany [1] using monovalent vaccines of Types O, A and C kindly furnished by IFFA, Lyon, where potency tests in cattle had been made with a high degree of statistical accuracy. Within each virus type, the vaccine represented by the left column had failed in the test in cattle, while that covered by the right column had passed. Figure 1 shows that the mouse test could distinguish the good from the poor vaccines within every virus type. As the height of the corresponding columns indicates, there was no complete quantitative agreement between the results of both tests. However, it should be remembered that, at that time, the mouse test was still being developed.

A synergic effect was observed in mice [2] with trivalent vaccine prepared by mixing equal quantities of the good monovalent ones (Nos. 2, 4 and 6 in Fig.1). The doses for mice were 0.5 ml of the monovalent and 0.25 or 0.5 ml of the trivalent vaccine. This means that mice inoculated with monovalent vaccine received 6 or 3 times more antigen of the respective type than those given trivalent vaccine. As Figure 2 shows, the results did not reflect this marked difference in dosage. The trivalent vaccine produced more Ab than expected, especially for Types O and A. It seems that, in this case, the C component boosted the O and A components, this effect being relatively strongest with Type O.

Later experiments made with other vaccine combinations showed that a synergic effect cannot be demonstrated in every case. The conditions under which it occurs are not precisely known. It seems, however, that the effect appears when a trivalent vaccine contains a monovalent one of especially high potency in mice.

The trivalent vaccines were not tested in cattle. According to a personal communication from Dr. Stellmann, IFFA, Lyon, a synergic effect is either missing or occurs very rarely in cattle treated with multivalent vaccines. It seems, therefore, that results obtained with such vaccines in mice are not comparable with those in cattle.

Table 2 shows results obtained in Ankara in comparative potency tests of monovalent Type O vaccines in mice and in cattle. The vaccines used were manufactured by Sap Institute, Ankara, and intended for use in the field. The tests in cattle were carried out by the late Dr. Ünlüeblebici. Serum pools from vaccinated mice were tested according to the old method, that is, undiluted and in serial two-fold dilutions against undiluted Ag. Results were also calculated as in earlier experiments, comparing mean percentages of unlysed cells in the serum-dilution range 1:1 to 1:32. The reference serum used in these tests originated from mice treated with vaccine 147 which was not very potent in cattle as indicated by the mouth lesions shown by all three vaccinated cattle upon intralingual challenge; foot lesions were missing in these animals.

It seems noteworthy that vaccines 160 and 164, which did not protect all cattle, also gave negative values in mice.

The data presented suggest that the mouse test can differentiate good and poor monovalent vaccines. Further work will have to be carried out to show whether or not it is suitable for quantitative potency tests. Due to the occurrence of a potentiating effect in mice inoculated with trivalent vaccine, the mouse test is not usable for such vaccines.

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1/ E. Traub, P. Thein and F. Kesting : A simple potency test for foot-and-mouth disease vaccines in mice and comparison of results with those of potency tests in cattle. Zbl. Vet. Med., B, 17, 1970, 389-400.

2/ E. Traub, P. Thein and F. Kesting : Antibody response in mice inoculated with monovalent or trivalent FMD vaccines. Zbl. Vet. Med., B, 17, 1970, 497-507.

Table 1

CHECKERBOARD TEST WITH SERUM POOL FROM MICE VACCINATED WITH BHK VACCINE TYPE O

Mixtures: 0.05 MS dil. + 0.05 Ag dil. (Frenkel Ag) + 0.1 buffered saline + 0.1 C' dil., 21 hs in refrigerator at +2°C; then 0.2 HS added; lysis at 37° for 60 min.

| MS | Ag kind | Ag dil. (1:..) | MS dil. (1: ...) 1 | 2 | 4 | 8 | 16 | SC 1 no Ag C' 1 | SC 2 no Ag no C' 1 |
|---|---|---|---|---|---|---|---|---|---|
| MS under test | $O_1$ | 1 | 100[1] | 62 | 25 | 0 | 0 | 0 | 100 |
| | | 2 | 94 | 75 | 50 | 25 | 0 | 0 | 100 |
| | | 4 | 87 | 62 | 37 | 12 | 0 | 0 | 100 |
| | | 8 | 25 | 12 | 6 | 0 | 0 | 0 | 100 |
| | | 16 | 6 | 0 | 0 | 0 | 0 | 0 | 100 |
| | N | 1 | 0 | 0 | 0 | | | | |
| | | 2 | 0 | 0 | 0 | | | | |
| | | 4 | 0 | 0 | 0 | | | | |
| Ref. | $O_1$ | 1 | 100 | 94 | 75 | 6 | 0 | 0 | 100 |
| | | 2 | 100 | 94 | 94 | 12 | 0 | 0 | 100 |
| | | 4 | 100 | 94 | 94 | 37 | 6 | 0 | 100 |
| | | 8 | 94 | 75 | 75 | 37 | 6 | 0 | 100 |
| | | 16 | 87 | 62 | 37 | 12 | 0 | 0 | 100 |
| | N | 1 | 0 | 0 | 0 | | | | |
| | | 2 | 0 | 0 | 0 | | | | |
| | | 4 | 0 | 0 | 0 | | | | |
| MNS | $O_1$ | 1 | 0 | 0 | 0 | | | 0 | 100 |
| | N | 1 | 0 | 0 | 0 | | | | |
| AC 1 no MS C' | $O_1$ | 1 | 0 | | | | | | |
| | N | 1 | 0 | | | | | | |
| AC 2 no MS no C' | $O_1$ | 1 | 100 | | | | | | |
| | N | 1 | 100 | | | | | | |

1/ Figures indicate estimated percentages of unlysed cells.

Table 2

VACCINE TESTS IN MICE IN COMPARISON WITH TESTS IN CATTLE AS PRACTISED BY SAP INSTITUTE

(monovalent Type O vaccines)

| Vaccine No. | Mortality from saponin | Tests in mice | | | Tests in cattle | |
|---|---|---|---|---|---|---|
| | | Results of CF tests | | | | |
| | | Serum pool from vacc. mice | Reference serum (Vacc.147) | Difference | Vaccinated cattle | Controls |
| 143 | 0/32 | 59[1] | 42[1] | +17 | 3/3[2] | 0/2 |
| 144 | 0/32 | 49 | 42 | + 7 | 3/3 | 0/2 |
| 146 | 1/32 | 53 | 42 | +11 | 3/3 | 0/2 |
| 147 | 0/32 | 42 | 42 | 0 | 3/3 | 0/2 |
| 155 | 1/32 | 44 | 34 | +10 | 3/3 | 0/2 |
| 156 | 0/32 | 47 | 34 | +13 | 3/3 | 0/2 |
| 157 | 8/38 | 53 | 30 | +23 | 3/3 | 0/2 |
| 158 | 10/32 | 28 | 21 | + 7 | 3/3 | 0/2 |
| 159 | 4/32 | 38 | 21 | +17 | 3/3 | 0/2 |
| 160 | 8/32 | 14 | 21 | - 7 | 2/3 | 0/2 |
| 161 | 0/32 | 43 | 21 | +22 | 3/3 | 0/2 |
| 162 | 2/32 | 31 | 30 | + 1 | 3/3 | 0/2 |
| 164 | 0/32 | 20 | 30 | -10 | 2/3 | 0/2 |

1/ Figures indicate mean percentages of unlysed cells in serum dilution range 1:1 to 1:32 (old method)

2/ No. protected/No. vaccinated.

Figure 1

# COMPARATIVE POTENCY TESTS OF MONOVALENT VACCINES IN CATTLE AND MICE

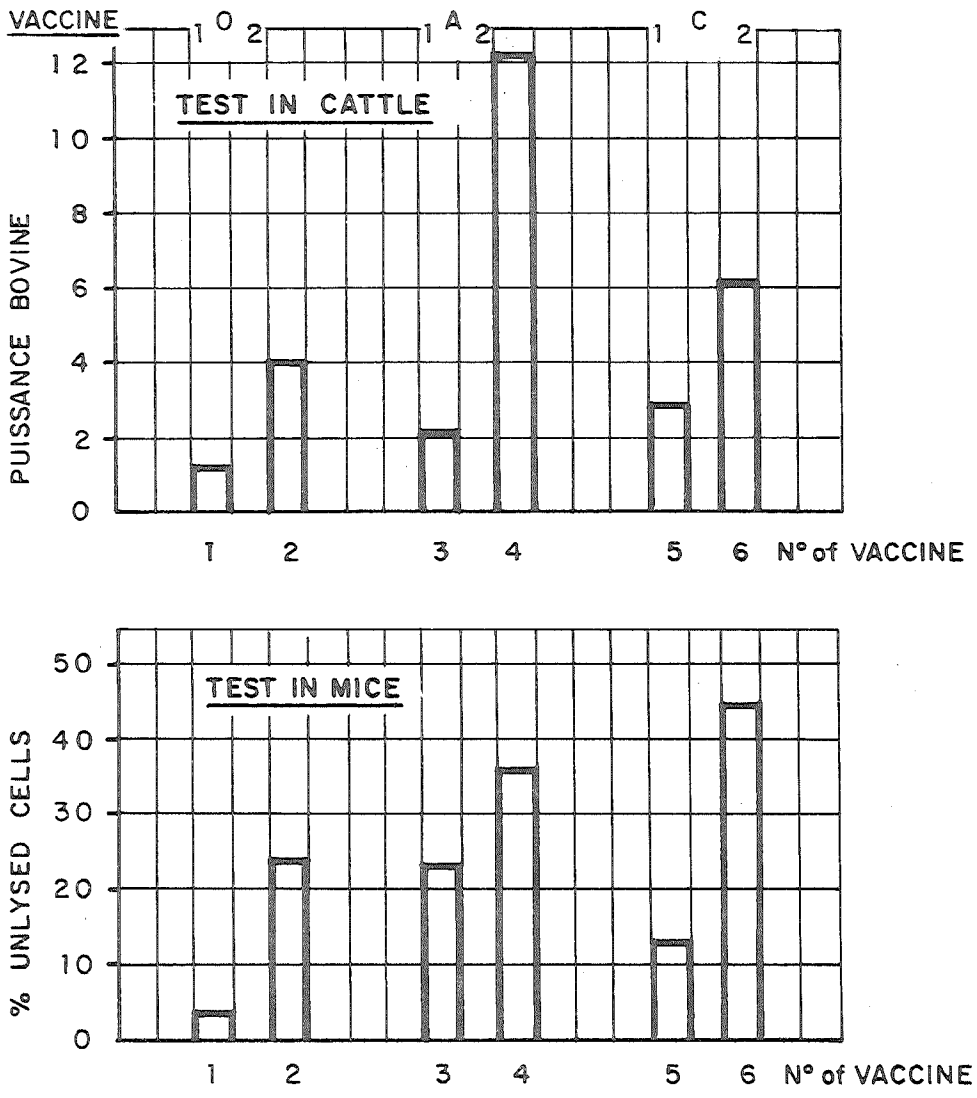
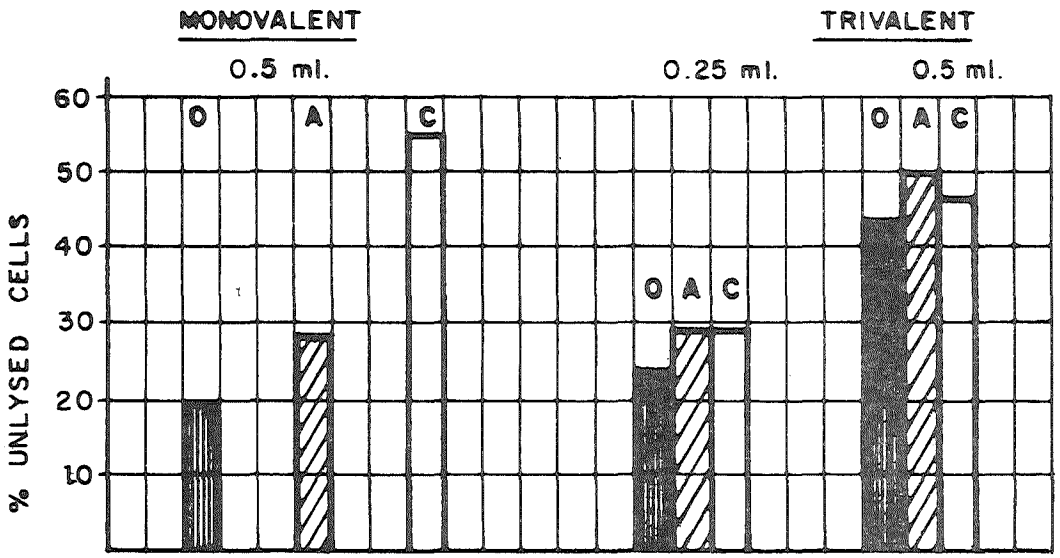


Figure 2

## SYNERGIC EFFECT IN TRIVALENT VACCINE



## Appendix 5

### A SEROLOGICAL STUDY OF 5 TURKISH TYPE A STRAINS

The purpose of this report is to describe the mode of reaction in crosswise complement fixation (CF) tests of 5 Type A strains isolated in Turkey in recent years and the extent of their cross-reaction with Type C.

### MATERIALS AND METHODS

The following virus strains were used:

(1) $A_{22}$, after several passages in cattle passed twice in BHK-21 cells and twice in BA cells;

(2) $A_{28}$, passage 11 in BHK cells after two passages in cattle. Strains $A_{22}$ and $A_{28}$ were prevalent in the country several years ago;

(3) A-Civril, obtained in 1970 from Western Turkey, 1st passage in BA cells after 2 passages in BHK cells;

(4) A-Izmir, from Western Turkey (1971) and passaged once in BA cells after two passages in baby mice;

(5) A-Van, originating in 1971 from an outbreak in Eastern Turkey near the Iranian frontier, $2^{nd}$ passage in BHK cells after one passage in baby mice;

(6) $O_1$, prevalent in Turkey for a number of years, passage 11 in BHK cells;

(7) $SAT_1$, no longer present in Turkey since 1966. For safety reasons, antigen was not made from this strain. Tests were carried out with inactivated guinea pig immune serum (GPIS) only.

All virus strains were obtained from the Serology Section of Sap Enstitüsü, Ankara, where they were established in cattle or baby mice.

For antigen production, the strains were grown in BHK-21 cells in 3-litre rolling bottles with Eagle-Earle growth medium and VM 3 (50 ml per bottle) as maintenance medium. Potent antigens, 500 ml per strain, were obtained in this manner.

In the tests presented in Table 2 and in Figure 2 of this appendix, infectious tissue culture fluid (TCF) served as antigen and was compared with two cell extracts prepared as follows: the detached cells from 10 rolling bottles per strain were sedimented by centrifugation, suspended in 25 ml VM 3, without lact-albumin hydrolysate and frozen for several days at -22°C; after thawing, the suspensions were cleared by centrifugation, the supernatant being the "1st cell extract" (CE 1), which served as antigen in the CF tests the results of which are recorded in Table 1 and in Figure 1; the sedimented cells were then ground intensively with VM 3 in Ten Broeck tissue grinders with well-fitting plungers to disrupt as many cells as possible; the volume of the ground cells was adjusted to 25 ml with VM 3; the turbidity remaining after centrifugation at 5 000 rpm for 20 minutes was greatly reduced by two cycles of freezing and thawing, so that it did not affect the CF tests; the final product, cleared by centrifugation, was designated "2nd cell extract" (CE 2).

For each virus strain, potent GPIS were prepared by applying one hyper-immunizing inoculation of guinea-pig passage virus into the foot pads about five weeks after primary infection; the virus contained 0.75 mg saponin/ml; the animals were exsanguinated from the heart seven to ten days after hyperimmunization.

Crosswise CF tests were carried out according to the checkerboard method; serum titres (serum dilutions showing 50 percent hemolysis) were recorded in the zone of antigen-antibody equivalence; none of the antigens tested were anticomplementary.

RESULTS

Mode of reaction of the A strains in crosswise CFT: Values of "R" calculated according to the formula now in general use 1/ are recorded in Table 1.

This table confirms that strains $A_{22}$ and $A_{28}$ are distinct sub-types and that $A_{22}$ and A-Izmir 1971 are practically identical. A-Civril 1970 occupies an intermediate position and strain A-Van 1971, although it cannot be considered a clear-cut sub-type conforming with adopted standards, sufficiently differs from the other strains to warrant special attention in a vaccination programme.

The mutual relationships of the various strains are more clearly evident from Figure 1 which shows that $A_{22}$ serum reacted with $A_{28}$ antigen more strongly than vice versa. This confirms earlier results obtained by the Serology Section of Sap Institute.

Relationship of the 5 A strains to other virus types: From Table 2 it can be seen that antisera against all five A strains cross-reacted to a greater or lesser degree with $O_1$ but not at all with $SAT_1$ antigen. From the fact that the second cell extracts shown in the last column gave by far the strongest non-specific reactions, it can be concluded that the common antigen is mostly cell-bound and that the bulk of it remains in the cells even after one cycle of freezing and thawing; mechanical disruption of the cells obviously liberates more non-specific antigen.

Figure 2 presents the result of a checkerboard test carried out with $A_{22}$ and $O_1$ antiserums and antigens. It illustrates the ratios of type-specific and non-specific antigens in infectious TCF as well as in the first and second cell extracts.

Contamination of the strains by a heterologous virus type seems most unlikely in view of the fact that all Type A strains and also $O_1$ strain gave evidence of a non-specific antigen. The latter is probably identical with the non-type specific antigen which has been known for a long time and which was studied in extenso by Cowan and Graves 2/ who designated it as "virus infection-associated (VIA) antigen" since it is not type-specific.

The non-specific antigen did not react with serial dilutions of a highly potent $SAT_1$ serum in the test listed in Table 2, but it did react with another $SAT_1$ serum prepared in the same way.

## SUMMARY AND CONCLUSIONS

Crosswise, quantitative complement-fixation tests carried out with five Turkish virus strains of Type A have shown that a strain identical with, or closely related to $A_{22}$ has recently reappeared in Western Turkey. At the same time, a Type A strain differing to a substantial extent from $A_{22}$ and $A_{28}$ has made its appearance in Eastern Turkey. The latter strain cannot be called a distinct sub-type but differs enough from the other strains to warrant attention in vaccination programmes.

A common, mostly cell-bound antigen was detected in five Turkish A strains and in the indigenous strain $O_1$. It appears to be identical with the VIA antigen studied by Cowan and Graves.

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1/ Joubert. L. and C. Mackowiak, 1968: La Fièvre Aphteuse. Fondation Mérieux, Lyon.

2/ Cowan, K.M., and J.H. Graves : A Third Antigenic Component Associated with Foot-and-Mouth Disease Infection. Virology 30, 1966, 528-540.

Table 1

VALUES (%) OF "R" FOR 5 TURKISH A STRAINS

| Strains | $A_{22}$ | $A_{28}$ | A Civril | A Izmir | A Van |
|---|---|---|---|---|---|
| $A_{22}$ | 100 | 31 | 75 | 100 | 63 |
| $A_{28}$ | 31 | 100 | 50 | 36 | 54 |
| A Civril | 75 | 50 | 100 | 66 | 66 |
| A Izmir | 100 | 36 | 66 | 100 | 55 |
| A Van | 63 | 54 | 66 | 55 | 100 |

$R \geqslant 90\%$ = serological identity

$90\% > R > 70\%$ = close relationship

$70\% > R > 32\%$ = more distant relationship

$R \leqslant 32\%$ = very distant relationship

$R = 0$ = no relationship

Table 2

PRESENCE IN BHK CELLS INFECTED WITH TURKISH TYPE A STRAINS OF A CELL-BOUND ANTIGEN CROSS-REACTING WITH TYPE $O_1$, BUT NOT WITH TYPE $SAT_1$

| Antigen[1/] | Antiserum | | Antigen dilution effecting 50% fixation | | |
|---|---|---|---|---|---|
| | | | Antigen: TCF | CE 1 | CE 2 |
| $A_{22}$ | $A_{22}$ | 1:80 | 1:11 | 1:24 | 1:22 |
| | $O_1$ | 1:80 | - | - | 1:12 |
| $A_{28}$ | $A_{28}$ | 1:100 | 1:11 | 1:44 | 1:11 |
| | $O_1$ | 1:80 | - | 1:3 | 1:6 |
| A Civril | A Civril | 1:100 | 1:11 | 1:11 | 1:10 |
| | $O_1$ | 1:80 | - | - | 1:3 |
| A Izmir | A Izmir | 1:120 | 1:11 | 1:21 | 1:11 |
| | $O_1$ | 1:80 | - | 1:1 | 1:5 |
| A Van | A Van | 1:120 | 1:11 | 1:23 | 1:13 |
| | $O_1$ | 1:80 | - | 1:2 | 1:10 |
| $O_1$ | $O_1$ | 1:80 | 1:6 | 1:19 | 1:11 |
| | $A_{22}$ | 1:80 | - | - | 1:6 |
| | $A_{28}$ | 1:100 | - | - | - |
| | A Civril | 1:100 | - | - | 1:1 |
| | A Izmir | 1:120 | - | - | - |
| | A Van | 1:120 | - | - | - |

- = no or less than 50 percent fixation in presence of undiluted antigen and all antigen dilutions

1/ all antigens gave completely negative reactions with $SAT_1$ antiserum in this and in a special "checkerboard" test.

Figure 1

# ANTIGENIC RELATIONSHIP BETWEEN 5 TURKISH TYPE A STRAINS

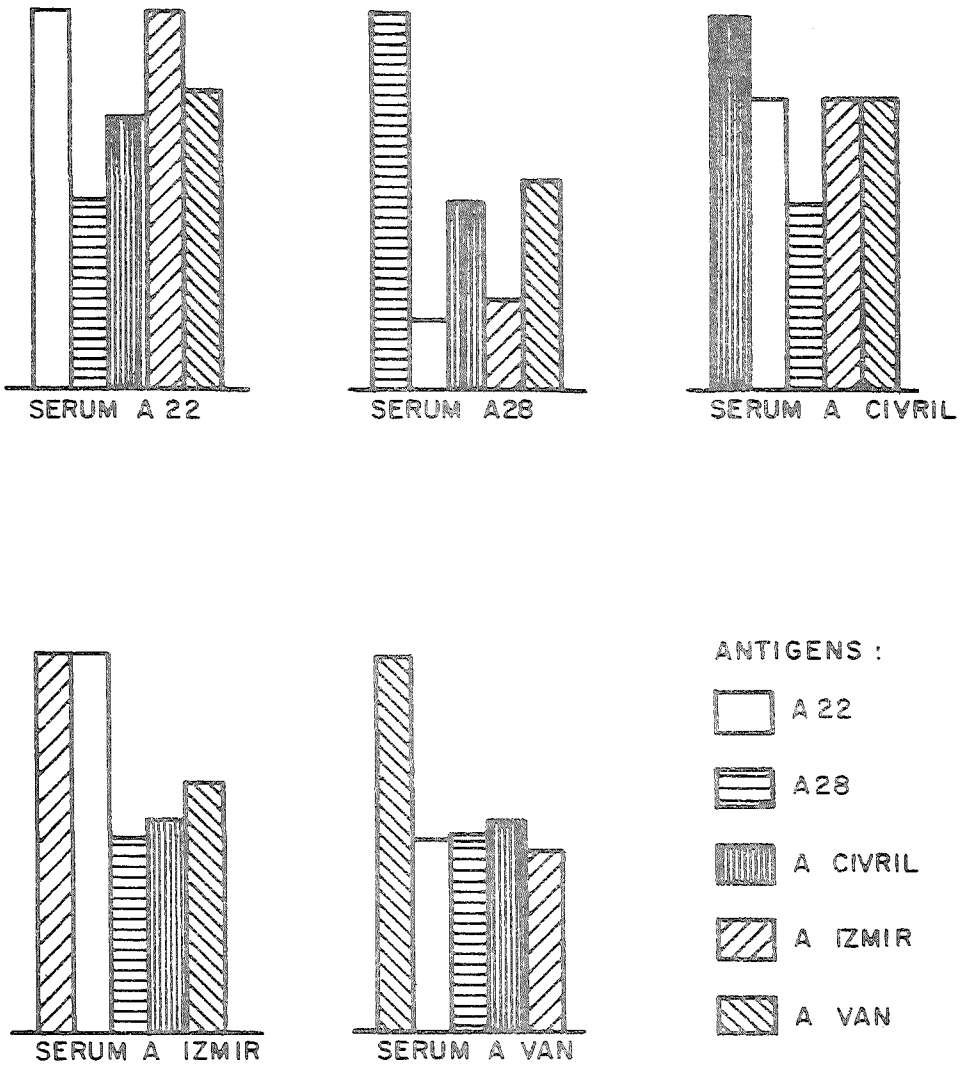
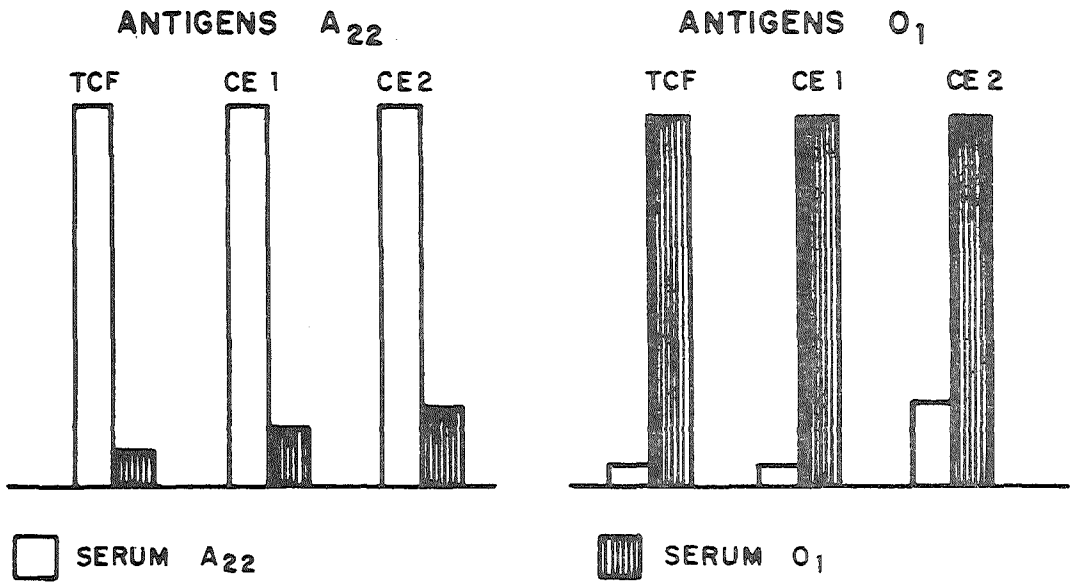


Figure 2

CROSS-REACTIONS BETWEEN $A_{22}$ AND $O_1$ SERUMS AND ANTIGENS IN QUANTITATIVE ("CHECKER BOARD") TEST.



MR/D0098/7.72/E/1/150