Arch. Inst. Razi, 1969, 21, 1 - 6

A NEW OVERLAY FOR PLAQUING ANIMAL VIRUSES (*)

by

HOSSEIN MIRCHAMSY AND FRED RAPP

During a previous study of African horse sickness virus (AHSV) (1), the plaquing efficiency of this virus under agar overlay was compared with the plaquing efficiency under methylcellulose (2) and starch gel (3). The low efficiency of plating under agar that has been observed for several viruses (4) was also noticed for AHSV. However, a marked increase in plaque number was observed when methylcellulose or starch gel were substituted for agar. Because of the technical difficulties in the preparation of the two latter overlays, a substance that was simpler to handle was sought as a substitute for agar. This report describes the successful development of an overlay employing tragacanth gum and reports the results of a comparative study of plaque formation under tragacanth and agar with foot and mouth disease (FMDV), vesicular stomatitis virus (VSV), pseudorabies virus, AHSV, and measles virus.

Materials and Methods. Cell cultures. Primary chicken ebmryo cells (CC) and the stable monkey kidney cell line (MS) were grown in 2-oz bottles in Earle's solution containing 0.5% lactalbumin hydrolyzate, 5% yeast extract (Difco), 10% calf serum, and 100 units of penicillin and 50 μg of streptomycin per ml. The baby hamster kidney cell line, BHK21 (5), and the porcine kidney cell line, BD (6), were also grown in 2 oz bottles in Hanks' balanced salt solution containing the same amounts of lactalbumin hydrolyzate, yeast extract, calf serum, and antibiotics as described above. All media were adjusted to a pH of 7.2-7.4 with 7.5% $NaHCO_3$.

The BSC-1 cells, a line derived from African green monkey kidneys, were grown in 60-mm plastic petri dishes in Eagle's basal medium, supplemented with 10% fetal bovine serum, and 10% tryptose phosphate broth. This medium also contained 100 units of penicillin and 100 μg of streptomycin per ml.

The petri dishes were incubated at 37° in an atmosphere of 5% CO^2. All cells were grown for 3—4 days at 37° before being used for experimental studies.

Virus. The VSV (Indiana serotype) was supplied by the Research Institute, Pirbright, Surrey, England and was already adapted to chicken cells (7). The pseudorabies virus was recently isolated by Dr. Sohrab in the State

(*) Reprinted from Proc. SOC. exp. Biol, and med. 1968, **129**, 13-17.

Razi Institute, Iran, and had been through 15 passages in MS cells and 5 passages in chicken cells.

The FMDV, types SAT1,O and A, were kindly supplied by Dr. Amighi, of the Foot and Mouth Disease Unit, Razi Institute, Iran. These viruses had undergone 5 passages in BHK-21 cells; type A was also subsequently adapted to BD cells through 11 passages. The AHSV, type 4, strain VRY, is a vaccine strain adapted to MS cells (8). The virulent Edmonston strain of measles virus is the same as that used in previous studies in this laboratory (9) and has been passaged numerous times in BSC-1 cells. The mouse adapted measles (MAM) virus had been obtained from Dr. Imagawa (UCLA) and had undergone 30-40 passages in suckling mouse brains (10).

Plaque assay. The growth medium was removed from cell monolayers grown in bottles and0. 2 ml of Tris saline was added to the cells. The virus, at various dilutions in Tris saline, was then added in 0.1 ml amounts into each of 3-4 bottles or petri dish cultures and allowed to adsorb for 2 hr at room temperature with occasional swirling of the cultures. At that time, the appropriate overlay was added, the cultures incubated at 37° and plaques observed as described in Results."

Tragacantlh overlay. Tragacanth gum (available from Fisher Scientific Company) is obtained from the small thorny shrubs of the various species of Astragalus grown in the desert regions of the Middle East. The white leaf gum of tragacanth (called ketira) obtained in Iran is the best grade on the market (11). It is a water soluble demulcent drug with a high molecular weight, and is used to coat irritated or abraded tissue surfaces to protect the underlying cells from irritating contacts (12). The gum consists of 60-70% bassorin, and 30-40% soluble gum or tragacanthin. Bassorin, consisting of complex methoxylated acids, resembles pectin and swells in the presence of water to form a colloidal solution. Tragacanthin yields glucuronic acid and arabinose when hydrolyzed. The main advantage is that tragacanth is not toxic for cells and in various experiments, it was found that animal cells under tragacanth overlay appear healthier and survive longer than cells under agar.

The tragacanth solution was made with a fine powder of white tragacanth that had been washed twice previously with ethyl alcohol and air dried. The powder was suspended in double distilled water (1.6 g/100 ml) at 50° with vigorous shaking. The suspension was autoclaved at 120° for 30 min. and then cooled to 37°.

To this suspension was then added an equal volume of warm (37°) double strength growth medium free of serum and phenol red. The pH was adjusted to 7.4 with 7.5% $NaHCO_3$ just before use. Six ml of tragacanth overlay was added to each bottle, which were stoppered and placed at 37°. With measles virus, a double strength Eagle's medium containing 10% fetal bovine serum was used. In all experiments, 100 units of penicillin and 100 μg of streptomycin per ml were included in the overlay. The petri dishes were incubated in 5% Co_2 at 37°. After 2-4 days, the overlay was removed and the cell sheets stained with 2 ml of a solution of tetrazolium salt (13). The dye was removed

from the cells after an incubation period of 2 hr at 37°, and plaques were counted after an additional incubation of 4 hr at 37°.

Agar overlay. The agar overlay was made by mixing equal parts of melted 2% agar (Difco Nobel) with double distilled water and media similar to those described above. The pH was adjusted to 7.4 with a solution of 7.5% $NaHCO_3$ before overlaying the cells. Six ml of agar overlay was added to each bottle. The bottles were inverted after the agar solidified, and incubated at 37° for 2—4 days. The cell cultures were then stained with 3 ml of solution of 1:7500 neutral red for 4 hr at 37°. For staining measles plaques, 5 ml of a second overlay similar to the first but containing 1:20,000 of neutral red was added on the fourth day and plaques were enumerated after overnight incubation at 37° in a CO_2 incubator.

Results. Comparison of plaque numbers under agar and tragacanth overlays. The comparative plaque counts obtained with various viruses under the different overlays are summarized in Table I. Clear and well defined plaques developed under the tragacanth overlay with all viruses tested. In most of the experiments presented, the number of plaques that developed was slightly higher under tragacanth than under agar. Significant differences in plaque numbers were not observed. Difference in plaque sizes were also noticed. The FMDV type A under agar yielded large plaques of 9 to 11 mm and small plaques of less than 1 mm in diameter (Fig. 1). The same difference was observed for plaques of VSV under tragacanth and under agar the latter were larger than the former. Measles plaques were also somewhat smaller under tragacanth than under agar.

Dose-response relationship. It was repeatedly noted that the number of plaques developing under tragacanth following inoculation of the appropriate cell culture with the various viruses was directly proportional to the concentration of the virus. To ascertain that the phenomenon followed a linear rela-

TABLE I. Comparison of Plaque Counts under Tragacanth and Agar Overlays.[a]

Virus	Cells	Period of incubation (days)	(pfu/ml)	
			Tragacanth	Agar
VSV (Indiana serotype)	Chicken	3	1.2×10^9	0.9×10^9
Pseudorabies	Chicken	2	3.2×10^8	2.7×10^8
FMDV (type SAT 1)	BHK 21	2	3.2×10^8	2.7×10^8
FMDV (type O)	BHK 21	2	2.8×10^9	2.0×10^9
FMDV (type A)	BHK 21	2	8.6×10^9	3.2×10^9
FMDV (type A)	BD	3	2.2×10^6	1.8×10^6
ASHV (type 4, strain VRY)	MS	4	3.5×10^7	1.8×10^7
Measles (Edmonston)	BSC-1	4	4.4×10^6	4.6×10^6
Measles (mouse adapted)	BSC-1	4	1.8×10^4	1.8×10^4

[a] Abbrev.: pfu = plaque-forming units; VSV = vesicular stomatitis virus; FMDV = foot and mouth disease virus; and AHSV = African horse sickness virus.

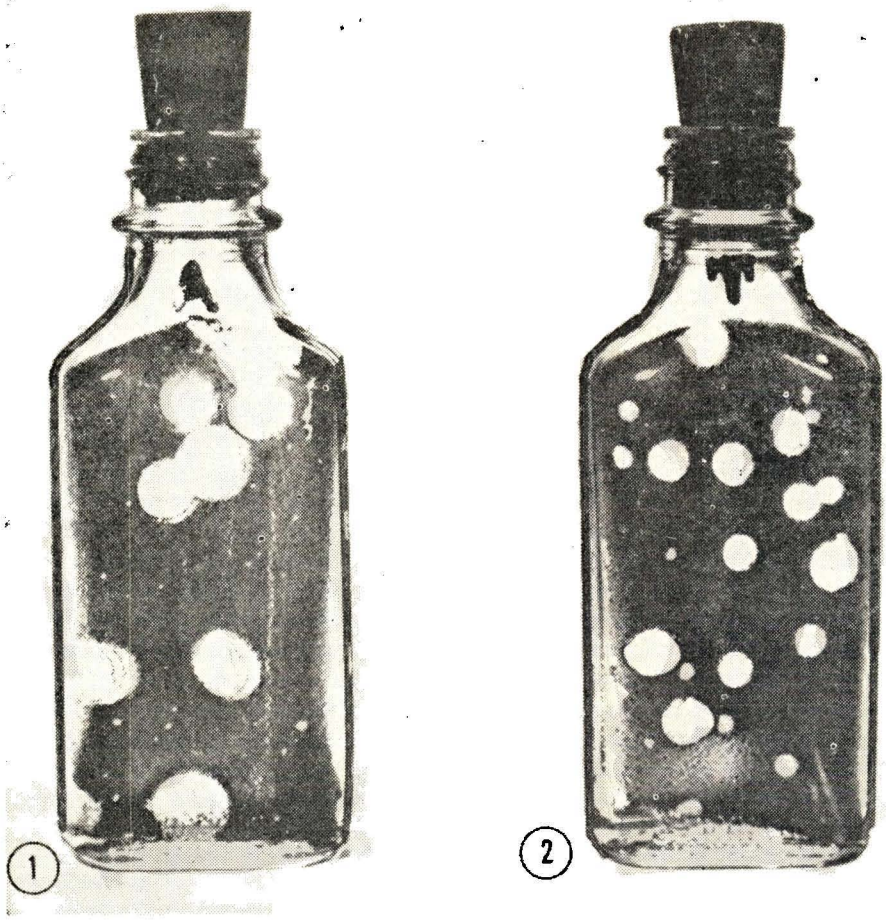


FIG. 1 (left) Plaques in BHK 21 cells produced by foot and mouth disease virus (Type A) under agar overlay 2 days after inoculation of the virus.

FIG. 2. (right) Plaques in BHK 21 cells produced by foot and mouth disease virus (Type A) under tragacanth overlay 2days after inoculation of the virus.

tionship, BSC-1 cell cultures were inoculated with various concentrations of measles virus. The virus was added in concentrations decreasing by a factor of 2-fold. The number of plaques developing was plotted against the virus concetration. As graphed in Fig. 3, it is evident that the development of measles virus plaques in BC-1 cells is directly proportional to the virus concentration used and that this relationship is a linear one.

Discussion. Our study with the viruses mentioned in this paper have shown that an overlay employing tragacanth instead of agar can be used with

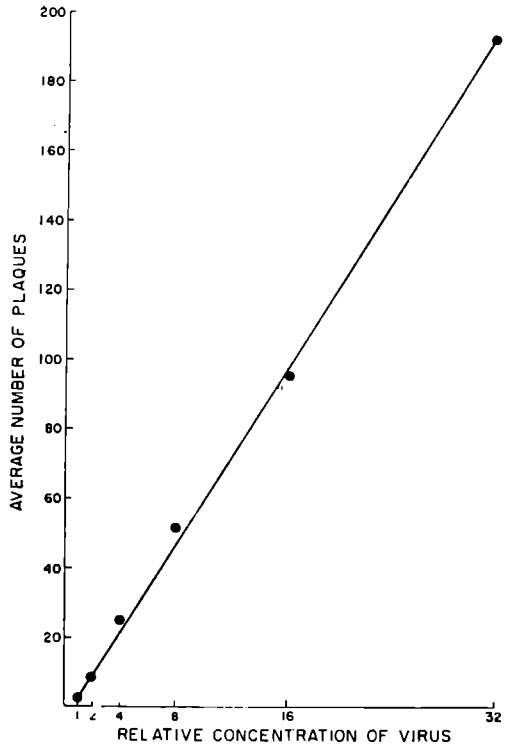


FIG. 3. Relationship of development of measles virus (Edmonston strain) plaques in BSC-1 cells to virus concentration under tragacanth overlay.

a variety of animal viruses. It is a matter of interest that all cell cultures used in this study looked much healthier under this new overlay than under agar; therefore, assays requiring long periods of incubation may be more feasible under tragacanth than under agar. We do not know whether the tragacanth is metabolized by the cells, but it is evident that the product is well tolerated by the cells used in this study.

Furthermore, tragacanth is easy to prepare and can be kept in the refrigerator for extended periods of time before use. Another technical advantage of tragacanth is that it can be easily removed for staining of the cultures. Preliminary experiments carried out in our laboratory have also revealed that cell cultures under tragacanth can be readily employed for the detection of virus antigens by the immunofluorescence technique. The well-known difficulty of removing either agar or methylcellulose from cultures does not appear to apply to the tragacanth overlay.

Summary. An overlay employing tragacanth gum as a substitute for agar in the plaque assay of animal viruses is described. The tragacanth overlay was successfully employed with vesicular stomatitis virus (a rhabdovirus), foot

and mouth disease virus (a picornavirus), pseudorabies virus (a herpes virus), African horse sickness virus, and measles virus (a paramyxovirus). Plaques under tragacanth were generally smaller but usually equalled or slightly exceeded counts obtained under the agar. The number of plaques developing with measles virus in BSC-1 cells was shown to be directly proportional to the virus concentration and the two variables were shown to have a linear relationship. Tragacanth is easy to prepare, the cells used tolerated long exposure to the material, and it was easier to remove than previously described overlays.

The authors thank Dr. Joseph L. Melnick for supporting and encouraging these studies.

1. Mirchamsy, H. and Taslimi, H., Can. J. Comp. Med. Vet. Sci. **30,** 47 (1966)
2. Hotchin, J. E., Nature **175,** 352 (1955).
3. DeMaeyer, E. and Schonne, E. Virology **24,** 13 (1964).
4. Cooper, P. D., Advan. Virus. Res. **8,** 319 (1961).
5. MacPherson, I. and Stoker, M., Virology **16,** 147 (1962).
6. Haag, J. and Santucci, J., C. R. Acad. Sci., Paris **120,** 1485 (1962):
7 Mirchamsy, H. and Cooper, P. D., Virology **20,** 14 (1963).
8. Mirchamsy, H. and Taslimi, H., Brit. Vet. J. **255,** 481 (1964)
9 Rapp, F., J. Bacteriol. **88,** 1448 (1964).
10. Imagawa, D. T. and Adams, J. M., Proc. Soc. Exptl: Biol: Med: **98,** 567 (1958):
11. Mantell, C. L., "The Water Soluble Gum." Reinhold, New York (1947).
12. Meyer, J. L., "Veterinary Pharmacy and Therapy," p. 48. Iowa State Univ Press, Ames, Iowa (1957).
13. Cooper, P. D.. Virology **7,** 469 (1959).

Arch. Inst. Razi, 1969, 21, 7 - 15

THE USE OF DRIED WHOLE BLOOD ABSORBED ON FILTER-PAPER FOR THE EVALUATION OF DIPHTHERIA AND TETANUS ANTITOXINS IN MASS SURVEYS (*)

by

H. MIRCHAMSY, F. NAZARI, C. STELLMANN, *and* H. ESTERABADY

Serological investigation of human and animal populations requires the use of blood sera. The serum, which is generally collected by venepuncture, cannot be obtained in large amounts from small children since the objections and emotional reactions of the parents are sometimes so strong that even small amounts of blood may not be obtained. The difficulties involved in the collection, storage and handling of small amounts of serum, when a very large number of specimens is to be tested, may also hinder the success of immunological studies of a large population.

On the other hand, it is desirable in small children to estimate the antibody level of childhood diseases, especially diphtheria and tetanus. To our knowledge, Taylor & Moloney[a] were the first to develop a microtechnique for estimating either diphtheria or tetanus antitoxins with as little as 0,1 ml of whole blood. This technique which is recommended by Zakarova et al.[b] in immunity determinations, also has the disadvantage of requiring proper diluents for the immediate dilution of blood, and again problems of storage and preservation of large numbers of small samples of blood exist. An easier method of collecting and storing large numbers of blood samples for evaluation of diphtheria and tetanus antitoxins is required. This is especially necessary in remote areas of developing countries. In the present report we describe a method of titration of antitoxins based on a technique using squares of filter-paper impregnated with whole blood obtained from finger-punctures for the titration of antibodies in several virus diseases.[c-i]

---

a Taylor, E. M. & Moloney, P. J. (1960) *Canad. J. publ. Hlth*, **51**, 135:

b Zakarova, M. S., Baeva, E. A. & Stepanova, N. A. (1963) *Z. Mikrobiol: (Mosk.)*. **40**, 68.

c Adams, E. & Hauson, R. P. (1957) *J. Bact.*, **72**, 572.

d Brody. J, A., McAlister, R., Haseley, R. & Lee, P. (1964) *J. Immunol:*, **92**, 954:

e Farrel, L. N., Reid, D. B. W. & Kormendy, A. C: (1963) *J: Immunol:*, **90**, 201:

f Gaggero, A. C. & Sutmoeller, P. (1965) *Brit. vet. J.*, **121**, 509:

(*) Bull wld Hlth org. 1968, **38**. 665-671

Materials and methods

Children under survey. Mass immunization of children against diphtheria and tetanus was started in Iran in 1950. The high level of fatalities from diphtheria fell markedly in the following years among immunized children. A further mass campaign was arranged by the Ministry of Health, Iran, in 1955. The first serological survey was conducted[j] in 1957 in Teheran when the Schick test was performed on 3209 children and the levels of diphtheria and tetanus antitoxins were studied in samples of venous blood collected from 683 children.

The present study was performed in a total of 20 maternity centres, kindergartens, primary schools and nursing centres in two cities (Isfahan and Shiraz). Blood was removed by finger-puncture from 2000 children previously immunized with triple vaccine (diphtheria-tetanus-pertussis (DTP) or combined diphtheria and tetanus (DT) vaccine.

As a rule, the children, randomly selected for this study, were immunized with 3 injections of DTP during the first 3 years of life and older children up to the age 14 years had received a booster dose of DT vaccine every 3-4 years. Details of the production of DT or DTP vaccines have been described in a previous report.[k]

Sources of specimens. In the preliminary experiment, the antitoxin titres of 10 children and of 60 guinea-pigs 300 g to 400 g in weight (immunized 12 months earlier with 2 doses of DT vaccine) were measured in samples of whole blood absorbed on filter-paper squares and compared with standard serum titrations; paired samples of venous blood and of finger blood absorbed on filter-paper were obtained simultaneously from each subject. The venous blood of children (and heart blood of guinea-pigs) was collected in standard Pyrex test-tubes (1.6 cm by 16 cm) and was allowed to clot at room temperature. The sera were separated from the clots, frozen and kept at a temperature of -25° for periods of 5 days to 2 months. The blood-saturated filter-paper squares were maintained at a temperature of + 25° C for up to 18 months before being tested.

Collection of blood on filter-paper. In the final field-trial, finger-blood of 1991 children was absorbed on to squares of filter-paper in the following way. Pieces of Whatman extra-thick filterpaper No. 31 (for chromatography) measuring 4 cm by 4 cm were stored in small envelopes (6 cm by 12 cm). The envelops with the enclosed filter-paper was sterilized in an oven at 100°C for 3 hours. The

---

g Green, R. H. & Opton, E. M. (1960) *Amer.* J. Hyg:, 72, 195

h Hendrickse, R. G., Montefiore, D., Peradze, T., Sherman, P. & Powell, M. (1966) *J. trop. Med. Hyg.*, **69,** 112:

i Karstad, L., Spalatin, J. & Hanson, R. P. (1957 *J. infect. Dis.*, **101,** 295.

j Mirchamsy, H., Nazari, F. & Sadegh, A. (1959) *Studies on diphtheria-tetanus.* In: *Proceedings. International Symposium of Immunology, Opatija,* Zagreb, p. 359.

k Mirchamsy, H., Taslimi, H. & Aghdachi, M. (1962) *Arch. Inst. Razi (Tehran),* **14,** 83.

child's finger was cleansed with alcohol and, when completely dry, was punctured with an ordinary lancet. A filter-paper square was held in a forceps and applied to the bleeding point gently and quickly until no white areas remained on the filter-paper. In preliminary experiments it was found that to cover the surface, each filter paper square absorbed 0.3 ml of blood; when several squares were required it was necessary to puncture a second finger. The squares were dried overnight at room temparture in the sealed boxes in the presence of anhydrous $CaCl_2$ and then stored at 4°C in the same boxes with anhydrous $CaCl_2$.

Serology. The blood was eluted from each square in 1.5 ml of peptone-saline the evening before the tests were to be run. Elution was carried out in test-tubes, the squares being soaked overnight at 4°C. It was accepted arbitrarily that each blood sample would contain 50% cells and 50% serum; hence the initial dilution was taken as a 1:10 serum dilution.

TABLE 1

RESULTS OF COMPARATIVE TITRATIONS OF DIPHTHERIA AND TETANUS ANTITOXINS FROM THE SAME CHILD USING SERUM AND WHOLE BLOOD ELUTED FROM FILTER-PAPER SQUARES

Child	Diphtheria antitoxin (AU/ml)		Tetanus antitoxin (AU/ml)	
	Serum titre	Filter-paper titre	Serum titre	Filter-paper titre
1	0.04	0.04	0.04	0.02
2	0.16	0.16	0.64	0.64
3	0.01	0.01	0.0025	0.0025
4	0.64	0.64	0.04	0.04
5	0.32	0.32	0.32	0.16
6	0.16	0.16	0.08	0.08
7	0.32	0.64	0.16	0.16
8	0.08	0.16	0.32	0.32
9	0.64	0.64	0.08	0.16
10	0.32	0.32	0.08	0.08

Each sample of eluted blood was tested for both diphtheria and tetanus antitoxins. Paired samples of squares kept up to 18 months have not shown a decrease in antitoxin titres when compared with squares submitted for titration soon after being prepared.

Diphtheria antitoxin assay. The technique used for assaying the diphtheria antitoxin was that described by Römer & Sames.[l] The matured toxin No. 560

---

l Roemer, H. P. & Sames, T. (1909) *Z. Immun.-Forsch.*, **3**, 344

m Ipsen, J. (1959) *J. Immunol.*, **83**, 448.

having a potency of 420 MILD/ml was used throughout these experiments. The L+ dose of this toxin was 1/20 ml. Both L+ and L+/100 were assayed; the volume of inoculum injected intradermally into 2 guinea-pigs was 0.2 ml in each assay. The range of concentrations used was doubled dilutions from 0.01/ml to 5.12/ml. When the two titres were not equal the lower titre was accepted as the final value.

Tetanus antitoxin titration. Titration of tetanus antitoxin was done with a technique developed by Ipsen,[m] testing doubled dilutions from 0.00126 AU/ml to 5.12 AU/ml. Local inbred mice were used. Tetanus toxin No. 11/65, precipitated by ammonium sulfate and dried, was diluted in 1% peptone-saline (10 g of Difco proteose peptone dissolved in distilled water up to a volume of 1000 ml. dispensed into 50-ml flasks and sterilized by autoclaving for 15 minutes at 15 $lb^{f}\text{-}in^{2}$; 1.05 $kgf\text{-}cm^{2}$), 0.55 mg of toxin being dissolved in 1000 ml of peptone-saline corresponding to 5 L+/1000 in 1 ml. The mixture of toxin and antitoxin was kept 1 hour at room temperature. Two mice were inoculated intramuscularly with the mixture. The mice were observed for 7 days and deaths were recorded each day. On the seventh day, the survivors without any signs of tetanus were considered as "normal" and those showing symptoms of tetanus as "with tetanus".

Results

Results of comparative assays for diphtheria and tetanus antitoxins using serum and filter-paper eluates from 10 children and 60 guinea-pigs are presented in Tables 1-3 and Fig. 1; it can be seen that in all cases the results were very similar and the differences never amounted to more than 1 dilution.

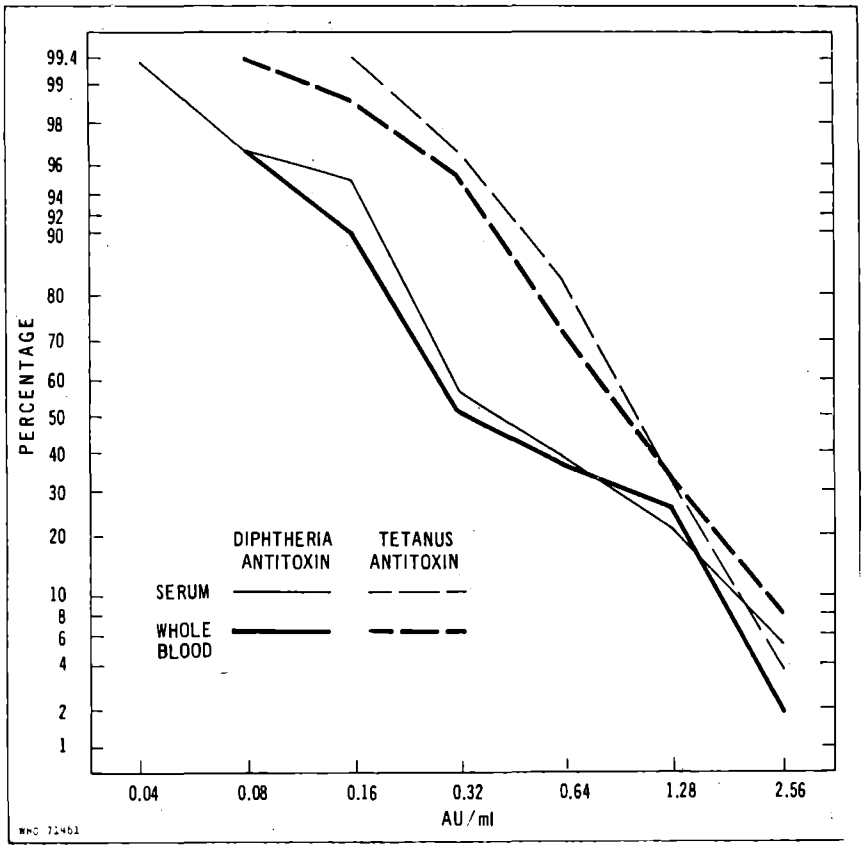
TABLE 2
COMPARISON OF DIPHTHERIA ANTITOXIN TITRES OF SERUM AND WHOLE BLOOD FROM 60 GUINEA-PIGS

	AU/ml							Geometric mean titre
	0.04	0.08	0.16	0.32	0.64	1.28	2.56	
Serum	2	1	23	11	10	10	3	0.35 AU/ml
Whole blood absorbed on filter-paper	2	4	23	9	7	14	11	0.32 AU/ml

TABLE 3
COMPARISON OF TETANUS ANTITOXIN TITRES OF SERUM AND WHOLE BLOOD FROM 60 GUINEA-PIGS

	AU/ml						Geometric mean titre
	0.08	0.16	0.32	0.64	1.28	2.56	
Serum	0	2	9	29	18	2	0.69 AU/ml
Whole blood absorbed on filter-paper	1	2	14	23	16	4	0.56 AU/ml

FIG. 1
CUMULATIVE PERCENTAGE OF DIPHTHERIA AND TETANUS ANTITOXINS DETERMINED IN SERUM AND ELUTED WHOLE BLOOD FROM 60 GUINEA-PIGS



The immune responses of 1991 children studied by means of the filter-paper technique are presented in condensed form in Tables 4 and 5. Table 4 shows that the booster dose of diphtheria antitoxin caused a relatively greater serological conversion rate in children of 7 years and more compared with children under the age of 7 years. This finding, which confirms the observation of earlier workers, may be interpreted as the boosting effect of natural contacts with carriers which increase gradually the antitoxin level in the circulation blood of older children. It may also be attributed to the great variations of immune response resulting from primary vaccination.

Discussion

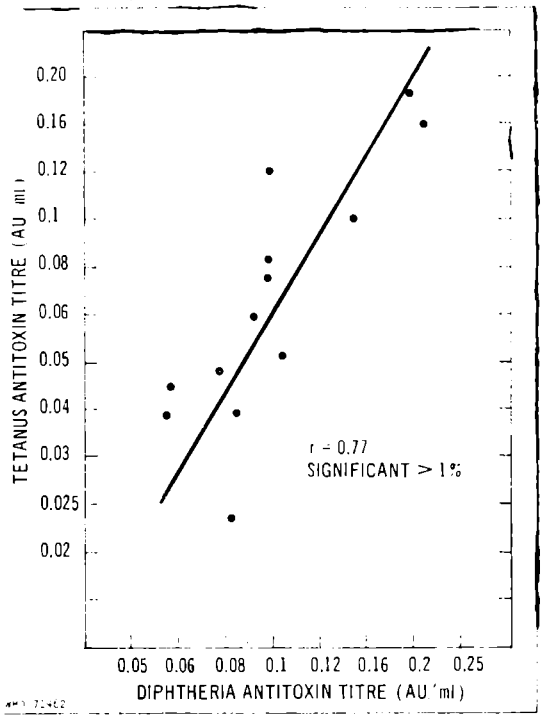
The serological conversion rates of 91% and 92% (Tables 4 and 5) are similar to our previous findings in which serum titration was carried out. The statistical

analysis of the data presented in Tables 2-5 is worth discussing briefly.

In Tables 2 and 3, the results of comparisons between serum titres and whole-blood titres of 60 guinea-pigs are summarized. From these tables it can be seen that the titres obtained by both methods are closely similar and in no instance was there a difference greater than 1 dilution between the titres of serum and of whole blood eluted from filter-paper.

FIG. 2

RRELATIONS BETWEEN TETANUS AND DIPHTHERIA ANTITOXIN TITRES IN THE SAME CHILDREN



Th antitoxin titres of 1991 children assayed with whole blood eluted from filter-paper square are given in Tables 4 and 5. The percentage of serological conversion is high. The geometric means of antitoxin titres and the limit of precision of the geometric means for each age-group show a marked increase in titres following the inoculation of each booster dose of bivalent DT vaccine; the homogeneous variations for different age-groups up to 16 years are statistically shown with a probability of 95% in Tables 4 and 5. These data show that the rise in titres of diphtheria and tetanus antitoxins in the same subject is parallel and

---

n Schwarz, D. (1963) *Méthodes statistiques à l'usage des médecins et des biologistes*, Paris, Flammarion.

TABLE 4

RESULTS OF DIPHTHERIA ANTITOXIN TITRES OF FILTER-PAPER ELUATES OF BLOOD SPECIMENS TAKEN FROM 1991 CHILDREN

Age (years)	No. of samples with following levels of titre (AU/ml):											Total	Percentage of immunity	Geometric mean (AU/ml)	Precision (fD) [a] ($\alpha = 0.95$)
	<0.01	0.01–0.02	0.02–0.04	0.04–0.08	0.08–0.16	0.16–0.32	0.32–0.64	0.64–1.28	1.28–2.56	2.56–5.12	5.12				
1	38	42	54	60	36	54	7	3	6	1	2	303	87.4	0.060	1.20
2	34	43	34	42	32	36	7	6	4	1	5	244	86	0.062	1.20
3	17	27	29	36	15	25	6	7	4	4	5	175	90	0.076	1.25
4	22	15	14	14	18	43	13	5	2	5	3	154	85.7	0.105	1.30
5	27	24	15	19	24	31	9	6	4	6	7	172	84.3	0.092	1.30
6	20	27	16	18	28	35	13	11	5	3	3	179	88.8	0.098	1.30
7	7	13	3	11	19	33	13	9	3	6	—	117	94	0.099	1.35
8	4	9	9	17	19	25	16	7	8	6	3	123	96	0.099	1.35
9	—	10	12	16	16	25	8	3	5	4	1	100	100	0.150	1.35
10	—	12	10	10	12	37	11	8	6	4	2	112	100	0.195	1.30
11	2	8	4	18	11	29	8	10	10	4	—	104	100	0.210	1.35
12	1	3	4	13	12	24	5	3	—	—	1	66	98	0.148	1.30
13	2	9	7	9	11	13	3	1	2	—	—	50	96	0.083	1.45
14-18	1	9	12	12	10	11	—	3	2	1	—	61	98	0.085	1.40
19+	2	3	2	2	2	5	2	—	—	7	6	31	93	0.400	2.30
Total	177	254	225	297	258	426	121	86	61	52	38	1931	91		

[a] The fD value should be multiplied or divided by the geometric mean value to obtain the confidence limits of the mean at a probability of 95 %.

TABLE 5

RESULTS OF TETANUS ANTITOXIN TITRES OF FILTER-PAPER ELUATES OF BLOOD SPECIMENS TAKEN FROM 1991 CHILDREN

Age (years)	No. of samples with following levels of titre (AU/ml):													Total	Percentage of immunity	Geometric mean (AU/ml)	Precision (fD) [a] ($\alpha = 0.95$)
	>0.0025	0.0025–0.005	0.005–0.01	0.01–0.02	0.02–0.04	0.04–0.08	0.08–0.16	0.16–0.32	0.32–0.64	0.64–1.28	1.28–2.56	2.56–5.12	5.12				
1	25	24	59	42	35	31	19	18	14	15	13	2	6	303	91	0.039	1.25
2	24	26	45	28	25	14	6	18	10	20	15	6	8	244	90	0.044	1.35
3	18	21	23	17	22	9	8	15	11	11	10	6	4	179	89.7	0.047	1.40
4	14	11	27	9	16	19	13	14	8	9	5	4	5	154	90	0.051	1.40
5	13	19	14	11	18	24	19	11	18	7	12	1	5	172	92	0.062	1.40
6	18	13	13	16	13	15	13	22	22	10	14	7	3	179	89.9	0.082	1.40
7	11	2	5	9	11	12	7	21	8	14	10	4	3	117	90	0.125	1.50
8	10	7	15	9	14	13	11	11	7	9	4	9	4	123	91	0.076	1.50
9	11	3	11	11	9	12	7	8	7	7	3	3	8	100	89	0.075	1.60
10	5	1	5	5	14	14	10	14	10	9	11	10	4	112	100	0.186	1.50
11	7	5	5	3	10	8	13	15	10	7	6	4	11	104	93	0.160	1.60
12	3	2	4	7	6	8	11	8	7	4	1	2	3	66	95	0.100	1.60
13	1	5	7	4	11	7	8	2	3	1	1	—	—	50	98	0.039	1.50
14-18	—	18	7	10	6	4	7	5	2	1	1	—	—	61	100	0.023	1.55
19+	—	9	10	5	3	2	1	—	—	1	—	—	—	31	100	0.069	2.80
Total	160	166	250	186	122	192	153	182	137	125	106	58	69	1 991	92		

[a] The fD value should be multiplied or divided by the geometric mean value to obtain the confidence limits of the mean at a probability of 95 %.

dependent on stimulating doses. By applying the statistical correlation of Schwarz[n] to the geometric means in Tables 4 and 5 for different age-groups we were able to obtain the coefficient of correlation ($r = 0.77$) for both antitoxins in the same subject (Fig. 2). This coefficient confirms once more our finding that the rise of both antitoxins after each booster injection of DT vaccine is parallel.

The valuable help of Dr Shahriari, Director General, Ministry of Health, Iran, is appreciated and the close collaboration of Dr Sotudeh and Dr Loghmani, Public Health Service of Isfahan, and Dr Petrossian, Public Health Service of Shiraz, in collecting blood samples is acknowledged.