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Egg Infectivity Assay of Trachoma Virus.* (28978)

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The report by T'ang *et al*(1) on successful isolation of trachoma virus in fertile chicken eggs has been confirmed(2). Growth of trachoma virus in the laboratory has contributed greatly to advancement of trachoma research. Before a virus can be studied in detail, a quantitative technique for its assay is necessary. Titration of trachoma virus in egg yolk sac is the only generally applicable method of assay. There are varying reports on reliability of yolk sac titration. Sowa and Collier (3) obtained irregular results and concluded that infectivity cannot be stated in terms of an egg 50% lethal end point. Jawetz and Hanna(4) reported that reliable and reproducible infectivity assays were usually obtained with an exception of seasonal insusceptibility of eggs during late summer and fall. Litwin(5) has stated that 50% lethal dose in eggs gives relatively poor estimates of total growth of trachoma. Based on experience with isolation of trachoma virus(6,7) where detection of virus depends on finding elementary bodies (EB) in smears of yolk sac membranes, usefulness of an egg-infectious titration based on demonstration of EB has been evaluated.

Materials and methods. Virus strains. Two strains of trachoma virus, TW-1 and TW-3 (formerly called TW-10 and TW-29), representing Taiwan immunological prototypes(8) and one, Bour(9), supplied by Dr. E. Jawetz were used in these studies.

Embryonated eggs. White shell eggs 50 ± g from White Leghorn chickens of the same stock fed on an antibiotic free diet were used exclusively. All eggs were incubated at 38°C before use.

Preparation of virus pools. Virus dilutions estimated to kill embryonated eggs in 7-9 days in 0.2 ml were inoculated into yolk sacs of 6- or 7-day-old embryos. Eggs were incubated at 35°C with air sac in upright position and candled daily. When about ⅓ had died, yolk sacs of remaining eggs were aseptically harvested. Attached yolk material was stripped away with forceps. Further removal of yolk was accomplished by placing harvested material at 4°C for ½ hour and then blotting on absorbent filter paper. Yolk sacs were pooled, weighed, and 20% suspension made

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TABLE I. Examples of Typical Egg Titrations of TW-1 and TW-3 Trachoma Viruses.

Virus strain	Dil of virus	Days of embryo death/ Elementary bodies + or −						Egg lethal dose (ELD_{50}) based on observation of 12 days	13 days	14 days	Egg infections Dose (EID_{50})
TW-1 (E_7)	10^{-1}	9+	9+	11+	13+	13+	13+				
	10^{-2}	11+	12+	13+	*+	*+	*+				
	10^{-3}	13+	14+	14+	*+	*+	*+				
	10^{-4}	(8)−	14+	*+	*+	*−	*−	$10^{2.0}$	$10^{2.9}$	$10^{3.4}$	$10^{5.0}$
	10^{-5}	*+	*−	*−	*−	*−	*−				
	10^{-6}	(10)−	*−	*−	*−	*−	*−				
TW-3 (E_3)	10^{-1}	6+	6+	7+	7+	7+	7+				
	10^{-2}	7+	8+	8+	10+	10+	10+				
	10^{-3}	8+	10+	10+	11+	11+	13+				
	10^{-4}	(5)−	11+	11+	12+	12+	13+	$10^{5.5}$	$10^{5.9}$	$10^{6.4}$	$10^{6.5}$
	10^{-5}	12+	12+	13+	14+	14+	*+				
	10^{-6}	12+	12+	*−	*−	*−	*−				
	10^{-7}	(5)−	*−	*−	*−	*−	*−				

* Embryo survived for 14 days.
( ) Embryos dying of non-specific causes are excluded from results.

in a modified sucrose-PG medium‡(10) by grinding in a sterile mortar and pestle. After light centrifugation, supernatant fluid was distributed in 2-5 ml amounts in screw capped vials and stored at −65°C in a mechanical freezer.

Egg titration. A vial of stock virus was rapidly thawed, 10-fold dilutions prepared in sucrose-PG containing 10 mg/ml of streptomycin, and kept in iced container until inoculated. Two-tenths ml of each dilution was injected by yolk sac route in five or six 6- to 7-day-old eggs which were then incubated at 35°C. All embryos dying during the first 3 days were discarded and remaining embryos candled daily for 13 days. Yolk sacs from each embryo that either died or survived within this period were smeared and stained by Macchiavello's method to determine the presence of EB. Embryo deaths prior to the sixth day of observation were rare. Deaths which occurred without demonstration of EB were considered non-specific and were excluded from results. Fifty per cent end points for infection (EID_{50}) based on EB in all eggs and for death (ELD_{50}) based on EB in dead eggs only were calculated by Reed and Muench method(11) and expressed as EID_{50} or ELD_{50} in 1 g of original yolk sac.

Results. Two examples of characteristic egg titrations of trachoma virus are given in Table I. The EID_{50} titers vary according to length of observation from 12 to 14 days. TW-1 virus killed embryos slower than TW-3 with dilutions of virus close to the limiting infectious dose. The difference between EID_{50} and ELD_{50} was greater with TW-1 virus.

To study the repeatability of egg titrations, 40 paired determinations of both ELD_{50} and EID_{50} have been performed on frozen pools of TW-1, TW-3, and Bour viruses. Table II shows that the average variation of paired titrations was considerably greater for the lethal dose than for the infectious dose. Storage of virus pools at −65°C caused little or no change of titer. Results shown in Table II include TW-1 and TW-3 pools tested after <1 and 17 months storage, and a Bour pool tested after <1 and 11 months storage. Variations observed during these long storage periods did not differ from those found when paired observations were closer in time, and the changes were not all falls in titer.

Table III shows that the difference between EID_{50} and ELD_{50} was consistently larger for TW-1 than for TW-3 and Bour. Egg passages 3 through 28 of TW-3, 7-24 of TW-1 and 12-18 of Bour were represented in these titrations. EID-ELD differences were similar for low and high numbered passages of the same strain. Also included were egg pools of TW-1 virus that had been reisolated

‡ Sucrose = 75 g; KH_2PO_4 0.52 g; Na_2HPO_4 1.22 g; l-glutamic acid 0.72 g; distilled water to make 1,000 ml; adjusted to pH 7.4 - 7.6 and autoclaved 10 lb for 15 min.

TABLE II. Variation in Repeat Egg Titrations of Frozen Pools of 3 Trachoma Strains.

Strain	No. of paired observations	ELD_{50} (Log_{10}) Avg titer	ELD_{50} Variation Range	ELD_{50} Variation Avg	EID_{50} (Log_{10}) Avg titer	EID_{50} Variation Range	EID_{50} Variation Avg
TW-1	13	3.6	0– .9	.4	5.4	.1–.8	.4
TW-3	14	5.4	.2–1.6	.8	6.5	0–.5	.2
Bour	13	5.7	.1–1.3	.6	6.7	.1–.5	.3
Total	40			.6			.3

Standard error of average variation for ELD_{50} was ± 0.067; for EID_{50}, ± 0.035.

from human and monkey experimental eye infections. The larger TW-1 EID-ELD difference was still present after primate passage. Pools of Bour virus made directly from conjunctival scrapings of severe monkey infections (although of low titer 10^1-10^2) showed the small EID-ELD difference characteristic of yolk sac pools of Bour. The closer the ELD_{50} approaches the EID_{50} the greater the lethality of the strain. TW-1 is less pathogenic for eggs than TW-3 and Bour. These results suggest that while it takes about 5 infecting doses of Bour and TW-3 to produce one lethal dose, it takes about 80 infecting doses of TW-1 to kill (based on 13 day incubation).

To evaluate the relationship between virus inoculum and time of embryo death, the average number of days required to cause death (A.D.D.) in titrations with the 3 strains was determined. The A.D.D. was determined only for dilutions of inoculum killing 60% or more of the eggs in 13 days. Live eggs were given a value of 14 days and included in the average. Relationships between A.D.D. and amounts of inoculum in EID_{50} are given in Fig. 1. A linear inverse relationship was obtained for each strain with log dilutions of virus (correlation coefficients of from 0.69 to 0.81, each significantly different from 0 at the $p < .005$ level by the t test). The lines are parallel with the slope of each closely similar (between —0.56 and —0.57 with a standard error <0.10). No matter what the dose level, eggs inoculated with TW-1 survived on average 2 days longer than those inoculated with TW-3 and Bour ($p < .005$ by t test). However, if the amount of inoculum is based on ELD_{50} a completely reverse result is seen with TW-1 killing embryos more rapidly than TW-3 and Bour. This is not unexpected because the concentration of inoculum for TW-1 is increased about 80-fold over the EID_{50} dose compared to a 5-fold increase for TW-3 and Bour. Although the relationship between ELD_{50} and A.D.D. was linear the lines were not parallel and because of the greater variability of ELD_{50} strain differences in day of death were statistically significant at a lower level ($p <$

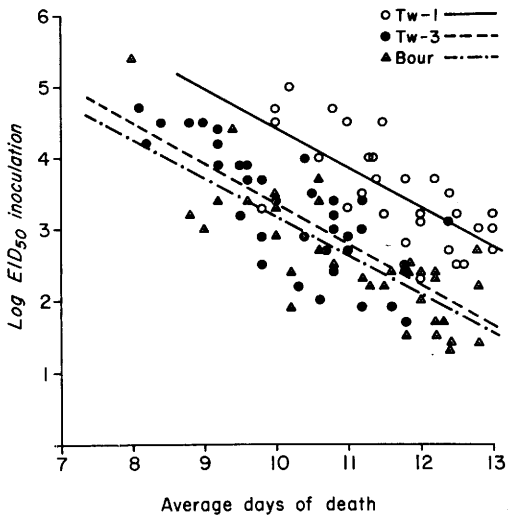

FIG. 1. Average day of chick embryo death with various inocula in EID_{50} based on a series of titrations of 3 trachoma strains.

TABLE III. Difference Between EID_{50} and ELD_{50} of 3 Trachoma Strains.

Strain	No. of titrations	EID_{50} — ELD_{50} (Log_{10}) Range	Mean (± S.E.M.)
TW-1	19	1.2–2.6	1.89 (± .08)
TW-3	20	0–2.0	.78 (± .15)
Bour	22	0–1.6	.70 (± .09)

EID_{50} — ELD_{50} = difference between EID_{50} and ELD_{50} in log_{10}.
S.E.M. = standard error of mean.

.05 by t test). Nevertheless, if ELD_{50} dose is used to measure inoculum, an incorrect conclusion would be reached that TW-1 strain is more lethal for eggs.

Discussion. This laboratory has had considerable experience in isolation of trachoma viruses. From Taiwan and India, 160 new isolates have been obtained, while 789 isolations have been accomplished from experimentally infected monkeys and humans. All these isolations have been based on demonstration of EB in yolk sac smears on first or after one blind passage. Whenever EB were seen, embryo death invariably occurred after additional passage demonstrating specificity of the microscopic findings. This extensive experience with yolk sac smears aided the precision of EID_{50} titrations. In these studies it was demonstrated that reproducible egg infectivity titrations can be obtained with 3 trachoma strains. An increase in reproducibility (from 0.6 to 0.3 $\log_{10}$ average paired difference) occurred when egg infectious titers were used instead of egg lethal titers, thus justifying the additional effort of examining smears of all eggs for EB. A recent report by Bell *et al*(12) shows EID_{50} titrations of SA-2 strain which are closely similar to those reported here with average variation in repeatability of 0.3 $\log_{10}$. Litwin(5) reported that compared to psittacosis viruses trachoma strains (TW-3 and Bour) grew much more slowly and were less lethal to chick embryos. ELD_{50} titer of trachoma virus depends largely on length of incubation period. When 6- to 8-day-old embryonated eggs are used, the longest practical incubation period is 13 days. Deaths of embryos caused by high dilutions of trachoma virus occur at the end of the incubation period while deaths from limiting infectious dilutions of psittacosis viruses occur a number of days earlier. Irregular deaths in higher dilutions decrease the repeatability of ELD_{50} titrations. We have not observed seasonal insusceptibility of eggs for trachoma virus.

Another example of the superiority of EID_{50} for trachoma virus is its better correlation with the minimal human infectious dose. Human volunteers were infected with 1 EID_{50} of TW-1(13) and TW-3(14). Bell *et al*(12) have reported similar results with the SA-2 strain. For all the trachoma strains tested the human infectious dose was less than 1 ELD_{50} and for the TW-1 strain 50-fold less. In addition, we have repeatedly infected monkeys with from 1 to 7 EID_{50} of Bour virus.

Even with the demonstrated linearity of the relation between EID_{50} and A.D.D. the single dilution method of titration used for psittacosis(15) is not applicable for precise titrations of trachoma virus due to the amount of scatter in days of death.

Difference in lethality among trachoma strains was found with Bour and TW-3 killing more readily than TW-1. It is not possible to say whether these findings are due to difference in rate of growth or to inherent lethality of strains. Reeve and Taverne(16) recently considered this question and concluded that in embryonated eggs all trachoma strains grow at the same rate and kill when the same total number of virus particles are reached. Differences among strains they attribute to differences in number of particles infective for the eggs. However, they did not determine the infectious dose but worked only with ELD_{50}'s. We find great variation in number of EB on smear from both dead and surviving eggs. In general, there are many fewer EB in eggs dying early (7 & 8 days) than in those dying later or surviving 13 days. Pools made from eggs dying early generally have a lower infectious titer than those dying later. If the extra effort to determine EID_{50} is not undertaken by laboratories willing to accept the increased variation of ELD_{50}, it is important to recognize that while the ELD_{50} titer gives a relatively close approximation of the infectious dose for strains like TW-3 and Bour, it gives a very false measure of the infectiousness of strains like TW-1.

Summary. Our method of egg infectivity assay for trachoma virus has been described. Because trachoma viruses grow more slowly in eggs (or are less lethal) than the related psittacosis viruses the highest dilutions which infect may not kill in 13 days. The egg infectious dose (based on demonstration of elementary bodies in dead eggs and those

surviving 13 days) gave higher titers and more reproducible results than the egg lethal dose. Strain differences in egg pathogenicity were demonstrated. It took 5 infectious doses of the Bour and TW-3 strains to produce one lethal dose, while 80 infectious doses of the TW-1 strain equalled one lethal dose.

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Persistent Infection of Human Cells (HeLa) with Adenovirus Type 12.* (28979)

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The oncogenic effect of human adenovirus type 12 (A-12) in newborn Syrian hamsters has been clearly established(1). Transformation experiments(2) with explants of primary human tissues were performed to determine the effect of A-12 on human tissues. It was found that few, if any, cells survived the cytopathic effects of the virus for extended periods. Although one of the major reasons for testing the oncogenic properties of the adenoviruses was their classical latency in human tissues(3), type 12 has been isolated only from fecal material and its possible role as a latent virus in human tissues has not been previously established.

To determine if methods could be found to prevent the extensive cytopathic effects of A-12 and to determine if a latent infection in human tissues could be established, a HeLa cell model system was employed. Particular attention was given to the serum components of the medium since Ginsberg reported that the establishment of carrier cultures with types 3 and 4 adenoviruses was dependent on an inhibitor fraction in human sera(4).

Materials and methods. Adenovirus type 12. Human adenovirus type 12, strain Huie, was obtained from American Type Culture Collection. The stock virus used was of the 8-10th passage in HeLa cells in this laboratory, and had a titer of approximately 2.5 $\log_{10}$ per 0.1 ml as determined by cytopathic effect on HeLa cells, with final reading on day 5.

Medium. Eagle's medium was used, with different sera and concentrations as described in the text. All sera were heat inactivated at 56°C for 30 minutes. Media contained 200

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