DEVELOPMENT OF A PASSIVE HEMAGGLUTINATION TECHNIQUE FOR ESTIMATION OF CHOLERA ANTITOXIN USING HUMAN O-Rh NEGATIVE ERYTHROCYTES AND PURIFIED CHOLERAGEN

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Section I

Objectives:

To quantitate anticholeragen by a simple, inexpensive and effective test for:

a. The estimation of antitoxin antibodies in the sera obtained from the participants in the toxoid field trial of Matlab Bazar using phase II lot 11201, Wyeth gluteraldehyde cholera toxoid.

b. The estimation of cholera antitoxin as well as the toxin by PHAI to support other future sero-epidemiologic or pathophysiologic research at CRL concerning cholera toxin and antitoxin.

This test should be as effective as the neutralization test on rabbit skin, should not necessitate decomplementation of sera and should not be complicated with non-specific agglutination due to heterophyle antibodies.

Section II

A. Budget Estimates:

a. Personnel:

Ansaruddin Ahmed	-Research Associate:	50%-total man-hour
P.K. Bose Neogi	-Senior Research Asstt.:	100% " "
O.G. Siddique	-Laboratory Technician :	50% " "
J.G. Gomes	-Laboratory Technician :	20% " "
S. Pashi	-Research Technician :	15% " "
S. Huda	-Research Technician :	10% " "

b. Equipment and Supplies:

c. Chemicals:

d. Usual illustration, secretarial and publication cost.

B. Research Plan:

a. Introduction:

1. Background and current status of work in the area.

Passive hemagglutination (will be referred as PHA subsequently) is now an accepted highly sensitive method of estimation of antibodies against various lipopoly-saccharide and protein bacterial antigens. The hemagglutination procedure, after its first description by Keogh et al. (1), has been improved much by investigators like Boyden (2) Stavitsky et al. (3) Neter (4) etc. Lee et al. (5) stressed the importance of micro modification of PHA technique using fresh and formalin-fixed human O-Rh negative erythrocytes. Ling (6) first mentioned the cell-stabilizing property of gluteraldehyde as well as its capacity of direct fixing of soluble proteins to erythrocytes without any additional coupling reagent. Bing et al. (7) also observed the same phenomenon.

The sensitivity of PHA is of the order of detecting 0.001 microgram of antibody nitrogen which is roughly equal to that of complement mediated bacteriolytic tests and toxin-antitoxin neutralization tests. It is about 20 to 100 times more sensitive than the corresponding bacterial agglutination and about 40 times more than the gel-diffusion (Kwapinski, 8).

Hochstein et al. (9) titrated cholera antitoxin by the PHA test, and found a high degree of correlation with the corresponding <u>in vivo</u> estimations, by using either fresh or formalin-fixed sheep erythrocytes and crude or purified toxin. Finkelstein and Peterson (10) achieved the same success by using tanned chicken erythrocytes as the innert particle.

2. Supporting Data:

Successful PHA techniques were set up in the CRL Immunology Laboratory in late 1969 as a preparation for offering serologic lab-support for future toxoid field trials at Matlab Bazar. Fresh sheep erythrocytes were sensitized with crude, partially purified (Spyrides; 11) and locally prepared highly purified toxin (prepared by Dr. A.K. Bhattacharjee) on the principle of conjugation by simple adsorption following the method of Hochstein et al. The results obtained using both crude, partially purified and highly purified toxin proved to be specific, sensitive, highly reproducible and of excellent correlation with the corresponding results obtained by Craig's (12) toxin neutralization tests done on rabbit skin.

3. Rationale:

Though the test mentioned above looked good it was not yet fit for supporting estimations of antitoxin titers in a large scale cholera toxoid field trial due to the following reasons:

(i) Non-specific agglutinations were found against non-sensitized sheep erythrocytes in a considerable number of cases. This was probably due to the occurrence of antigen-antibody reactions associated with Forssman type of heterophile antigen-antibody system.

It is almost impossible to adopt the method of absorption of sera with sheep erythrocytes to obviate this problem when a laboratory is to handle thousands of tinny amounts of finger-prick sera.

(ii) Decomplementation of sera prior to the PHA test was necessary to prevent complement-mediated lysis because of the use of fresh sheep erythrocytes. An alternative method was sought in which the erythrocytes would be stabilized against lysis by the use of chemicals like aldehydes.

(iii) The use of crude or partially purified toxin as the sensitizing antigen might lead to false positive results because of its heterogenous nature as we know crude culture filtrate of Inaba 569B gives at least 4 precipitin bands in immunodiffusion test against the corresponding anti sera. Methods were tried to obviate these problems both by using Dr. Finkelstein's purified lyophilized toxin and Wyeth toxoid as well as by using other types of erythrocytes like human O-Rh negative, chicken, goose and gander cells.

b. Materials:

1. Sensitizing antigens: Dr. Finkelstein's purified lyophilized choleragen Lot No. 0572 having 27 lb/microgram (according to the titrations done at CRL and at Brooklyn by Dr. J. P. Craig) and Wyeth toxoid Lot 11201 were used for coating erythrocytes.

2. Standard antiserum used as reference for calculating antitoxin unitage of unknown sera: Lyophilized Swiss Serum and Vaccine Institute (SSVI) Standard antitoxin Lot No. EC3(A-2/67)-B containing 4470 Craig AU/ml.

3. Indirect particles: Fresh, tanned, formalin and gluteraldehyde fixed erythrocytes of the species chicken, gander, goose and human (Ø group, CDE negative type) were used according to the requirement of the respective methods.

4. Antisera tested:

(i) Two anti-whole cell (washed) cholera vaccine and 2 anti-purified ogawa antigen hyperimmune rabbit sera were tested for checking the specificity of the test.

(ii) A panel of 36 lyophilized immune antitoxoid human sera from volunteers of the H-8 Texas Study who were boosted at 26 weeks were tested undiluted and at an initial dilution

of 1:10 for the estimation of cholera antitoxin.

(iii) About 6700 samples comprising pre- and post-immunized finger-prick sera (considered to be 1:10 saline-diluted plasma) along with their appropriate controls (DT toxoid injected as placebo) obtained from the participants of the phase II Wyeth Cholera Toxoid Field Trial at Matlab Bazar were tested for estimation of antitoxin.

c. Methods:

(i) Finkelstein and Peterson's method of simple adsorption of purified choleragen and choleragenoid on tanned chicken erythrocytes was tried.

(ii) Simple adsorption of purified choleragen and choleragenoid was tried on gluter-aldehyde fixed gander, goose and human erythrocytes according to the test conditions of Hochstein et al. (9) with slight modifications.

(iii) The method of direct coupling as done by Stavitsky and Arquilla (3) using the bivalent reagent bis-diazotized benzidine (BDB) was tried with fresh and gluter-aldehyde fixed erythrocytes.

(iv) Gluteraldehyde was used as a bivalent reagent by a modified method of E. Onkelinx et al. (13) performing direct coupling of proteins to erythrocytes probably by stable covalent linkages as with reagents like BDB. The same three types of erythrocytes were used as particles.

(v) Chromium chloride was used as a coupling reagent with the same types of erythrocytes as above following the method of Vyas and Shulman (14) for coating human 'O' cells with hepatitis associated Australia Antigen.

(vi) Titrations of cholera antitoxin by the rabbit intracutaneous method for comparison and correlation with the PHA titrations were carried out by Dr. Al Mahmood according to a recent set of methods and instructions by Dr.Craig (15).

a. Results:

(i) The test by Finkelstein and Peterson's method using choleragen was of lower sensitivity than the test by simple coating of crude toxin on sheep cells. The control chicken cells showed weak autoagglutinability. There was no agglutination of cells coated with Wyeth toxoid against any antitoxin.

(ii) Simple adsorption of choleragen on gluteraldehyde-fixed human erythrocytes was effective at a lower order of sensitivity as compared to that of the chromium chloride and gluteraldehyde coupling methods.

(iii) The method using BDB had to be discarded due to the occurrence of partial hemolysis even after stabilization of the erythrocytes with gluteraldehyde.

(iv) Coating of toxin on human erythrocytes with gluteraldehyde proved to be one of two best methods in this laboratory the other being using chromium chloride as the coupling reagent. Gander cells, though satisfactorily sensitized with toxin with the help of gluteraldehyde, were discarded due to a few non-specific agglutinations with control cells.

Figure 1 shows that both the methods, namely by the use of gluteraldehyde and chromium chloride, bear good correlation with each other and with the corresponding rabbit intracutaneous neutralization test when the antitoxin unitages of the panel of 36 hyperimmune human sera from H-8 volunteers were determined in reference to the SSVI standard antitoxin. The almost complete

parallelism of the two calculated correlation lines stresses the fact that either of the two methods could be used with equal facility for estimation of cholera antitoxin.

Non-sensitised control erythrocytes in both the tests showed no non-specific agglutination in any of the 36 sera at 1:2 and 1:20 final dilutions.

The anti-whole cell vaccine and the antipurified ogawa lipopolysaccharide hyperimmune rabbit sera did not show any agglutination with the sensitized erythrocytes in either of the methods.

(v) Figure 2 shows an excellent correlation of results (r=0.970) obtained at CRL using the gluteraldehyde method with those obtained at Dr. Peterson's laboratory when the same panel of sera from H-8 volunteers were tested. (Data was kindly sent by Dr. Johnny W. Peterson as a personal communication to Dr. W.F. Verwey). The antitoxin contents of 9 samples were not detectable by both laboratories and one sample was not detectable by the CRL Laboratory probably because of higher initial dilution to start with (1:20 final dilution in the 1st well after adding sensitized cell suspension).

(vi) The results of antitoxin titration of about 6700 finger-prick serum samples from the participants of Phase II Wyeth Toxoid Field Trial will be presented in a separate report by Dr. George Curlin.

(vii) Figure 3 shows a correlation of antitoxin units/ml of 53 selected toxoid field trial samples by rabbit skin method versus the number of tubes obtained in titration by gluteraldehyde PHA. Samples having different levels of antitoxin titers ranging from just above the average lowest level of detection by PHA to a very high level were selected and this correlation was examined in retrospect. The figure does not include the data for the sera having antitoxin contents below the detectable level of this PHA technique.

Discussion

The low sensitivity of Finkelstein and Peterson's method in our experiment and the weak autoagglutinability of the non-sensitized chicken erythrocytes was probably due to the un-suitability of the lot of tannic acid used. Variation of effects of different lots of tannic acid have been noted in many laboratories.

The low order of sensitivity of the test using simple adsorption of toxin on gluteraldehyde-fixed cells may probably be explained by the highly purified nature of toxin and the use of erythrocytes other than sheep cells.

Non-specific agglutination of goose and gander cells may be associated with a possible presence of heterophile antigen in them as duck erythrocytes have been reported to contain heterophile antibodies (15) Humphrey and White.

The hemolysis found in the use of BDB is a well known fact due to the damage this reagent produces on the cells.

The lowest level of antitoxin detected by PHA technique with finger-prick field serum samples delt in the particular method of titration and the initial dilution corrected for the average hematocreit value was approximately 4.9 AU/ml; but if a sample is added undiluted in the 1st well of microplate and the doubling dilution by microdilutor starts from the 2nd well a lowest detectable level of 2.45 AU/ml could easily be reached. Starting with undiluted serum from venous blood in the 1st well the lowest detectable level will go down to approximately 0.326 AU/ml.

The reason for selecting the gluteraldehyde-coupling method over the equally effective method using chromium chloride was that gluteraldehyde stabilized the erythrocytes in addition to conjugating choleragen protein on their surfaces. Thereby, it obviates the chance of hemolysis either by complement mediating or by detergent contamination of the microplates, Since decomplimentation of sera and the control runs of non-sensitized cells were not necessary we could streamline the hemagglutination procedure to titrate about 6700 serum samples from the toxoid field trials in 28 working days with the help of two technicians!

A correlation having a coefficient value of 0.97 between the results obtained at the CRL Laboratory for the same panel of sera with those obtained independently in a competentlaboratory like that of Dr. Peterson's puts this newly developed PHA method in good confidence.

FIGURE 1

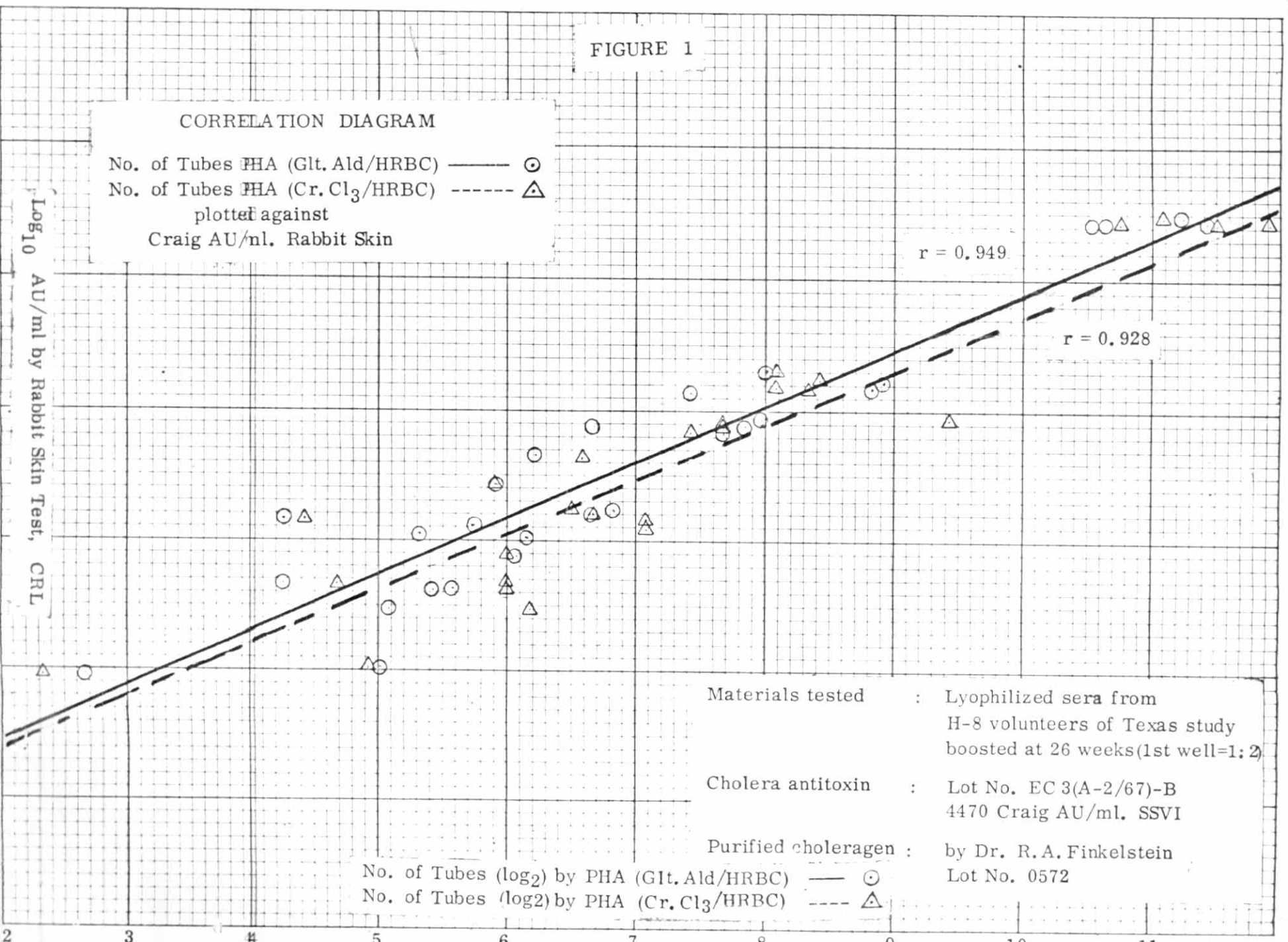
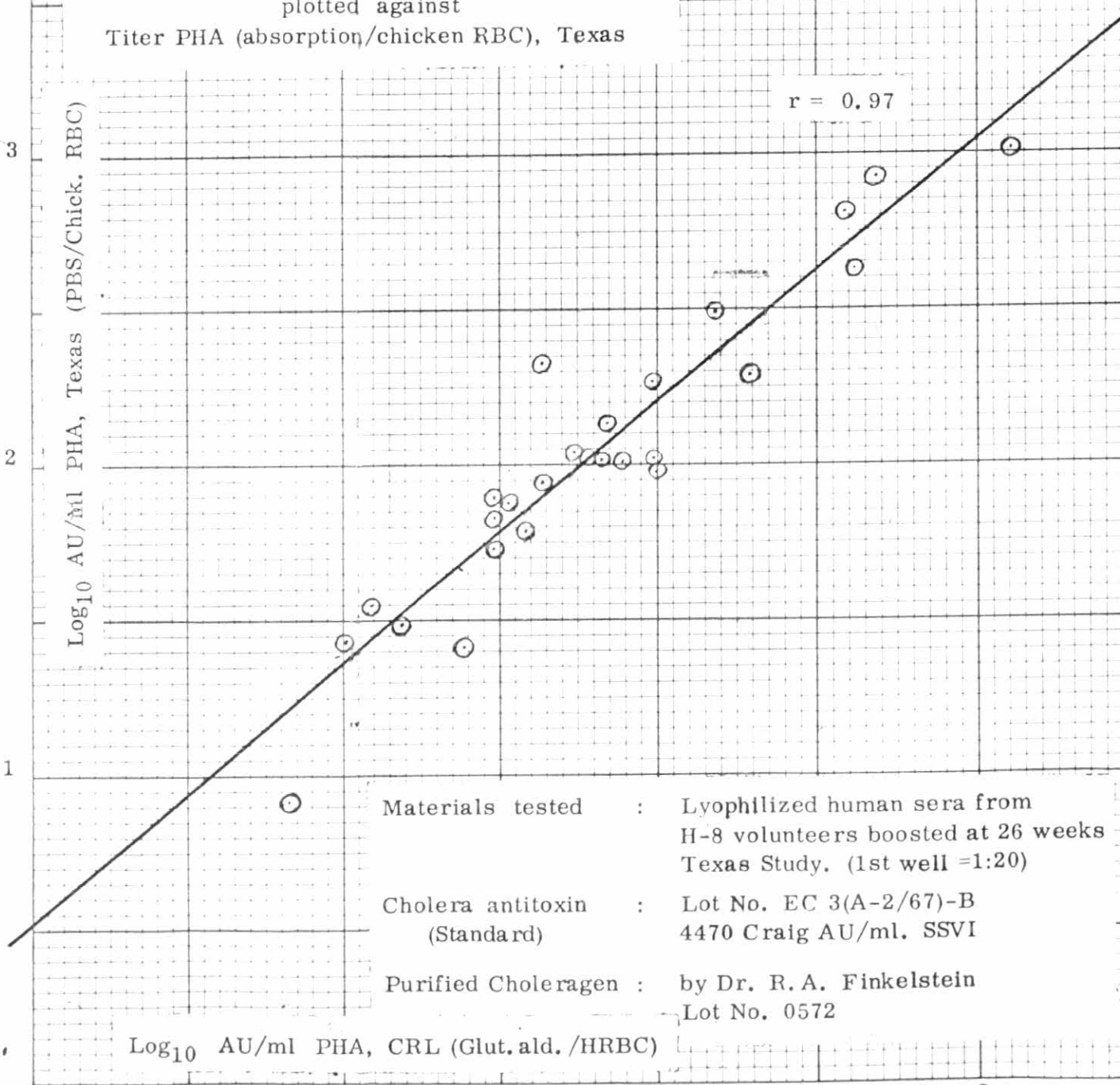

CORRELATION DIAGRAM
No. of Tubes PHA (Glt. Ald/HRBC) ——— ⊙
No. of Tubes PHA (Cr. Cl3/HRBC) ------ △
plotted against
Craig AU/ml. Rabbit Skin
r = 0.949
r = 0.928
Log10 AU/ml by Rabbit Skin Test, CRL
Materials tested : Lyophilized sera from H-8 volunteers of Texas study boosted at 26 weeks (1st well=1:2)
Cholera antitoxin : Lot No. EC 3(A-2/67)-B 4470 Craig AU/ml. SSVI
Purified choleragen : by Dr. R.A. Finkelstein Lot No. 0572
No. of Tubes (log2) by PHA (Glt. Ald/HRBC) —— ⊙
No. of Tubes (log2) by PHA (Cr. Cl3/HRBC) ---- △
2
3
4
5
6
7
8
9
10
11

FIGURE 2

CORRELATION DIAGRAM
Titer PHA (gluteraldehyde/O'Rh-ve HRBC), CRL
plotted against
Titer PHA (absorption/chicken RBC), Texas

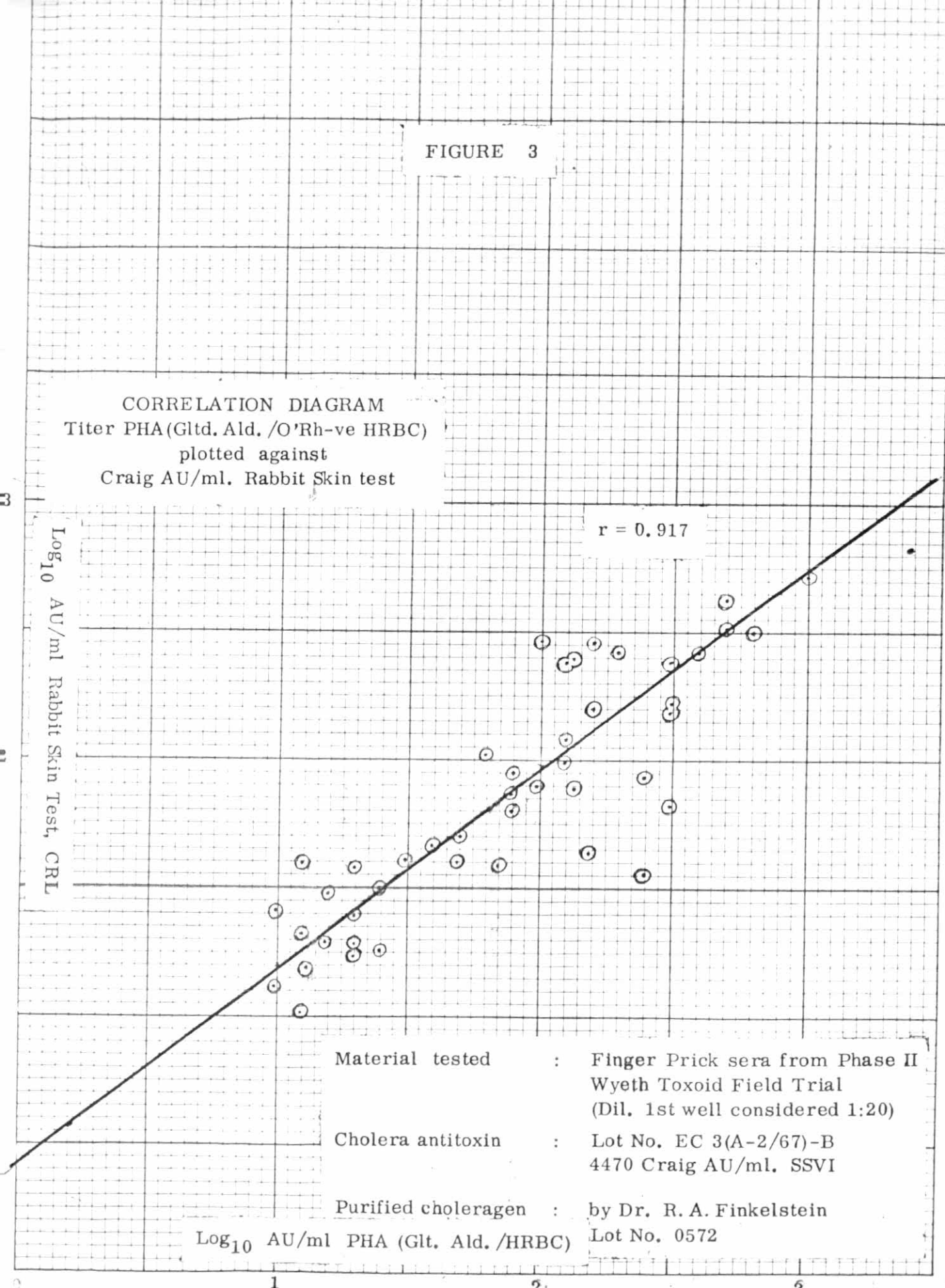


Materials tested : Lyophilized human sera from H-8 volunteers boosted at 26 weeks Texas Study. (1st well =1:20)

Cholera antitoxin (Standard) : Lot No. EC 3(A-2/67)-B 4470 Craig AU/ml. SSVI

Purified Choleragen : by Dr. R. A. Finkelstein Lot No. 0572

FIGURE 3


CORRELATION DIAGRAM
Titer PHA (Gltd. Ald. /O'Rh-ve HRBC)
plotted against
Craig AU/ml. Rabbit Skin test
r = 0.917
Log10 AU/ml Rabbit Skin Test, CRL
Log10 AU/ml PHA (Glt. Ald. /HRBC)
Material tested : Finger Prick sera from Phase II Wyeth Toxoid Field Trial (Dil. 1st well considered 1:20)
Cholera antitoxin : Lot No. EC 3(A-2/67)-B 4470 Craig AU/ml. SSVI
Purified choleragen : by Dr. R. A. Finkelstein Lot No. 0572

PROCEEDINGS OF THE 9TH MEETING OF THE SCIENTIFIC REVIEW AND TECHNICAL ADVISORY COMMITTEE OF THE CHOLERA RESEARCH LABORATORY

(a)

and

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