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FACTORIAL EXPERIMENTAL DESIGN APPLIED TO THE IMMUNOLOGICAL STUDY OF TWO FOOT-AND-MOUTH DISEASE VIRUS SUBTYPES. 1. A GREECE 1969 — A ALLIER EXAMPLE

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Résumé

PLAN D'ANALYSE FACTORIELLE APPLIQUE A L'ETUDE IMMUNOLOGIQUE DE DEUX SOUS-TYPES DE VIRUS APHTEUX. — 1. Une épreuve immunologique croisée double pratiquée par la méthode de l'indice K est soumise à une analyse statistique d'expérience factorielle. Les virus A Grèce et A Allier, qui ont été pris comme exemple de la marche à suivre dans les calculs, apparaissant comme deux sous-types immunologiquement différents.

Foot-and-mouth disease vaccines prepared in a given country are aimed at protecting bovine against certain viral subtypes. The emergence of a new subtype of virus, revealed by a serological test, clearly raises the question of the risk presented by this new subtype. Answer to this question can only be provided by a double immunological cross-test, the principle of which is as follows :

With viruses 1 and 2, suspected of being of 2 different subtypes, 2 vaccines Va 1 and Va 2 are prepared, and each of these vaccines is tested against each virus, Vi 1 and Vi 2, in the following way :

Va 1 :
Vi 1 (homologous reaction)
→ Efficiency E 11
Vi 2 (heterologous reaction)
→ Efficiency E 12

Va 2 :
Vi 2 (homologous reaction)
→ Efficiency E 22
Vi 1 (heterologous reaction)
→ Efficiency E 21

In each of the 4 cases thus defined, efficiency E of the vaccination will be interpreted by values either rore or less different from one another : E 11, E 12, E 227 E 21. This efficiency E will be appreciated with methods usually employed to check the efficiency of unknown vaccines :

— Protection percentage against podal generalisations
— Protective dose at 50 % (or vaccine potency)
— K protection index

The result of the immunological test will be provided by the comparison of homologous

and heterologous reactions and can be outlined as follows :

1 — Viruses 1 and 2 will be considered as constituting the same subtype,
if : E 11 = E 12 and E 22 = E 21.

2 — Viruses 1 and 2 will be considered as belonging to 2 different subtypes,
if : E 11 > E 12 and E 22 > E 21.

3 — Viruses 1 and 2 will be considered as belonging to 2 different subtypes, virus 1 being predominant,
if : E 11 ≃ E12 and E 22> E 21.

A. Factorial design principle

The double immunological cross-test is essentially comparable to a factorial experiment 2 × 2, where 2 factors A and B are each studied at 2 levels : 1 and 2. In fact, it consists of studying the action of 2 factors : one, the vaccine, the other, the virus. Each one can be considered at 2 levels : virus 1 level and virus 2 level.

The advantage of the factorial design of experiment lies in the fact that it enables what is called factor interaction to be studied and its significance to be tested. For example, if it is a question of 2 medicaments A and B administered at 2 levels (presence and absence) the 4 combinations capable of being administered will be :

AO :
medicament A, no medicament B
OB :
no medicament A, medicament B
OO :
no medicament A, no medicament B
AB :
medicament A, medicament B

Study of the interaction consists of contrasting (AO + OB) with (OO + AB). This therefore represents the effect that cannot be explained by isolated effects of the 2 medicaments A and B. According to whether this interaction is positive or negative, the physiologist will say that B potentiates the effect of A, or that B inhibits the effect of A. In other words, a positive interaction signifies that actions A and B are not just additives.

With the double immunological cross-test, the 4 treatments are represented by the following efficiencies :

E 11 (vaccine 1, virus 1),
E 12 (vaccine 1, virus 2),
E 22 (vaccine 2, virus 2)
and E 21 (vaccine 2, virus 1).

The discovery of the interaction will consist of contrasting (E 11 + E 22) with (E 12 + E 21), or in other words, comparing all the homologous reactions with all the heterologous ones. The term « interaction » of the factorial analysis will therefore constitute the test, revealing the existence of 2 different subtypes, by making a global analysis and not a separate one treating E 11/E 12 on the one hand and E 22/E 21 on the other

A factorial design is applicable when each animal from the 4 groups provides a quantitative immunitary response. Among the methods usually employed for checking vaccines, K index (Lucam *et al.*, 1958) presents this advantage. In fact, testing of vaccinated animals involves a virus titration in 20 sites, by an intradermo-lingual route. Each vaccinated animal provides an individual V or Vi titer which is its own, and enables an individual K or Ki index to be calculated, which is the measurement of its immunitary state.

We have
Ki = T/Vi similarly to K = T/V
T represents the titer of the same virus calculated on non-vaccinated control animals.

Lastly, to fully appreciate the risk presented by the emergence of a new subtype, it is important to carry out these tests at an immunitary level which is not too far removed from the minimal efficiency required for the vaccines :
log. K = 1.2.
Vaccine doses are therefore scaled in geometrical progression, so as to investigate an immunity area including the value K = 1.2.

We have had the opportunity of applying this factorial design to the study of several pairs of immunolgical subtypes. In the first part of this work, we will keep to only the pair A Greece 69 — A Allier, and will examine in detail the factorial analysis which was carried out on this occasion. In the second part of the study, we propose to make a series of hypotheses regarding situations that may theoretically be anticipated, and to examine the behaviour of different factor analysis terms. We will be able to note that most of the anticipated

situations have in fact been realised and that the 5 pairs of subtypes which have been studied by this method illustrate perfectly the different hypotheses that can be formulated regarding immunological subtypes.

B — A Greece 69 — A Allier example

METHODS AND RESULTS

Towards the end of 1969, outbreaks of foot and mouth disease appear in Greece, provoked by a type A strain. Serologically, this A Greece 69 virus differs from the A Allier virus used as antigen in French vaccines. The problem of knowing whether the Greece strain could present a danger, should it emerge in France, arose.

1. Methods.

Six groups of 4 bovines were formed and labelled A, B, C and A', B', C'. With the Allier vaccine, groups A and A', B and B', C and C', were vaccinated with vaccine doses scaled in geometrical progression (pure, 1/4 and 1/16 of the dose). Three weeks later, the K indices of the A, B, and C groups were established with the Allier virus (homologous test AA) and those of groupes A', B' and C' with the A Greece virus (heterologous test AG).

The second part of the experiment was constituted by 6 other groups of 4 bovines which were vaccinated with scaled doses (1, 1/4, 1/16) of A Greece vaccine, prepared by a French Institute of production. Three weeks later, 3 groups were tested with the A Greece virus (homologous test GG) and the others with the A Allier virus (heterologous test GA).

For each of the 12 experimental groups, the

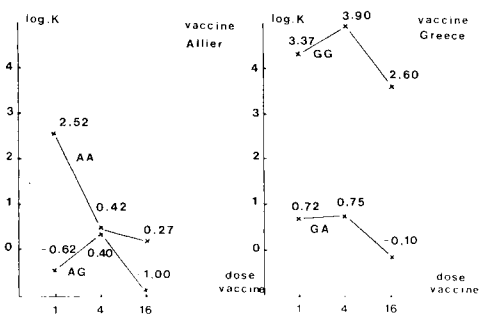

Fig. 1. — Results of the double immunological cross test (A Greece 69 — A Allier).

K value and the individual K value for each of the 4 bovines forming a group, were calculated.

2. Results

The results of the group K indices have been represented in the 2 parts of figure 1.

For the Allier vaccine, the homologous AA response was all in all greater than the heterologous AG response. The result was similar for the Greece vaccine (GG> GA). One of the vaccines (Greece vaccine) was better than the other. The decrease of efficiency induced by the heterologous reaction seemed to be less important in the case of Allier vaccine. The scaling of the vaccine doses has not given very satisfactory results in the sense that the immunitary response to 1/4 of the dose was often better than those obtained with the full dose.

The aim of the factorial analysis will be to find out if the differences noted were significant. Calculations were performed according to Lison (1958), using the author's notations.

C. Factorial analysis

Table 1 presents the necessary data for the calculations.

1. Variance analysis

The statistical analysis consists firstly of making a global study of the effect of the treatments (AA, AG, GG and GA) and that of the vaccines doses (1, 1/4 and 1/16). We will therefore calculate the sums of the corresponding squares :

Correction term :

$$1\,010^2/48 = 21\,252$$

Total sum of squares :

$$35\,112 - 21\,252 = 13\,860$$

Treatments sum of squares :

$$[249^2/12] + [71^2/12] + [515^2/12] - [175^2/12] - 21\,252 = 8\,989$$

Sum of squares for doses :

$$[400^2/16] + [379^2/16] + [231^2/16] - 21\,252 = 1\,060$$

Error :

$$13\,860 - (8\,989 + 1\,060) = 3\,811$$

The sums of the squares thus obtained enable us to draw up a general variance analysis table (see table 2).

The F tests carried out on the mean squares

Table 1. — Immunological test A Allier — A Greece 69 (individual values of K).
In the first column of each section feature the values Ki ; in the second column, one finds the data again after simplification (multiply by 10 and add 10 ; for example 1.9 → 19 → 29). Finally, the totals for the sections, the rows and the columns appear in the various table-margins.

Dose of vaccine	Vaccine: Allier — Challenge: Allier (AA)	Vaccine: Allier — Challenge: Greece (AG)	Vaccine: Greece — Challenge: Greece (GG)	Vaccine: Greece — Challenge: Allier (GA)	Mean for each dose
1	1.90 29 841 3.10 41 1681 1.20 22 484 3.90 49 2401 SX = 141 SX² = 5407	−1.00 0 0 −1.00 0 0 −1.00 0 0 0.50 15 225 SX = 15 SX² = 225	4.00 50 2500 4.00 50 2500 1.50 25 625 4.00 50 2500 SX = 175 SX² = 8125	0.90 19 361 −0.30 7 49 1.20 22 484 1.10 21 441 SX = 69 SX² = 1335	m1 = 25.00 SX = 400
1/4	0.50 15 225 1.00 20 400 −0.30 7 49 0.50 15 225 SX = 57 SX² = 899	−1.00 0 0 1.50 25 625 0.60 16 256 0.50 15 225 SX = 56 SX² = 1106	4.00 50 2500 4.00 50 2500 4.00 50 2500 3.60 46 2116 SX = 196 SX² = 9616	1.10 21 441 2.50 35 1225 −0.30 7 49 −0.30 7 49 SX = 70 SX² = 1764	m1/4 = 23.68 SX = 379
1/16	1.20 22 484 0.50 15 225 −0.30 7 49 −0.30 7 49 SX = 51 SX² = 807	−1.00 0 0 −1.00 0 0 −1.00 0 0 −1.00 0 0 SX = 0 SX² = 0	4.00 50 2500 1.60 26 676 2.40 34 1156 2.40 34 1156 SX = 144 SX² = 5488	0.10 11 121 −0.30 7 49 −0.30 7 49 0.10 11 121 SX = 36 SX² = 340	m1/16 = 14.43 SX = 231
All doses	SX = 249 SX² = 7113 mAA = 20.75	SX = 71 SX² = 1331 mAG = 5.91	SX = 515 SX² = 23229 mGG = 42.91	SX = 175 SX² = 3439 mGA = 14.58	All data SX = 1010 SX² = 35112 n = 48 m = 21.04

of treatments and doses reveal that the effects of the treatments and those of the doses were highly significant.

2. Regression effect/log dose

This part of the calculations is aimed at seeing whether there exists on the whole a relation between K values and vaccine doses. To do this, we will attribute conventional values (1, 2 and 3) to the doses, in accordance with table 3, and we will calculate the sums of the necessary squares while studying the regression.

$Sx^2 =$

$16\,(1^2 + 2^2 + 3^2) - [(6 \times 16)^2/(3 \times 16)] = 32$

$Sxy =$

$(1 \times 400) + (2 \times 379) + (3 \times 231) - [(1\,010 \times 6 \times 16)/(3 \times 16)] = -169$

$Sy^2 = 1\,060$

Variation due to the regression :

$[(Sxy)^2/Sx^2] = [(-169)^2/32] = 892$

Deviation from linear regression :

$1\,060 - 892 = 168$

Table 4 shows us that the regression is highly significant and, henceforth, we can calculate the mean regression coefficient.

$b = [Sxy/Sx^2] = -169/32 = -5.3$

(that is, -0.53 after return to original data)

Table 2. — First analysis (without considering interaction) of the data of table 1.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	Significance
Total	13860	47			
Treatments	8989	3	2996	33.20 * * * P < 0.01	$F^3_{42} = 6.60$ (P = 0.001)
Doses	1060	2	530	5.88 * * P < 0.01	$F^2_{42} = 5.15$ (P = 0.01)
Error	3811	42	90	—	

Table 3. — Computation of the sums of squares required for the regression study.
(X = doses of vaccine ; Y = immunological response K).

Parameters	Dose of vaccine			Sums
	1	1/4	1/16	
X	1 × 16	2 × 16	3 × 16	6 × 16
Y	400	379	231	1010

Table 4. — Variance analysis of the regression of K on the dose of vaccine.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	Significance
Regression	892	1	892	9.91 * * P < 0.01	$F^1_{42} = 7.27$ P = 0.01
Deviation from linear regression	168	1	168	1.86	N.S.
Error	3811	42	90		

Table 5. — Computation of the sum of squares by the polynomial coefficient method.

Main effects	AA	AG	GG	GA	S (KT)	[S (KT)]²	rSK²	Sum of squares	Degrees of freedom	F	Significance
Interaction	+1	−1	+1	−1	518	268324	12 × 4	5590	1	62.11** P < 0.01	$F^{1}_{42} = 7.27$ P = 0.01
Vaccine	+1	+1	−1	−1	370	136900	12 × 4	2852	1	31.68**	
Virus	+1	−1	−1	+1	162	26244	12 × 4	546	1	P < 0.01 6.06** P < 0.05	$F^{1}_{42} = 4.07$ P = 0.05
Error								3811	42		

The line plotted on figure 2 represents the regression of the protection index K on the dose of vaccine.

The connection is significant and linear only when the 4 regressions AA, AG, GG and GA are considered as a whole. The fact that certain individual regressions are not linear does not prevent further inferences, because the factorial analysis could be carried out in the same way if the 12 animals from each group had received the same vaccine dose.

3. Analysis of main effects and interaction.

The sums of corresponding squares are calculated by the polynomial coefficient method and different stages of the calculation are set out in table 5.

This table enables us to interpret the main effects.

a) The homologous reactions induce an immunity greater than that of the heterologous reactions. Test F indicates a highly significant effect. It can therfore be concluded that different subtypes are involved.

b) Whatever the test virus, the Greece vaccine induced greater immunity than the Allier vaccine. Tests F indicates a highly significant effect. It can therefore be concluded that the Greece vaccine has shown itself to be better than the Allier vaccine.

c) Whatever the vaccine, the Greece virus test revealed greater immunity than that of the Allier virus. Test F only indicates a significant effect. The Allier virus shows a certain dominance compared with the Greece virus (at least in respect of vaccines and doses utilised).

After returning to original data, the average immunities obtained for each of the 4 groups (AA, AG, GG, GA) are represented in figure 3. The slope of the regression lines is that calculated before, i.e. −0.53. It is a mean slope which comprehensively represents the regression of immunitary responses according to the dose. Figure 3 has the advantage of being a simplification of figure 1.

4. Simple effect analyses

These comparisons of experimental groups 2 to 2, were carried out with the t test and we will firstly calculate the standard deviation of the difference from the mean square of the error :

$$s_d = \sqrt{(2 \times 90)/12}$$

With t = 2.02 or 2.70 for 42 degrees of freedom we will have :

$$t.\ s_d = 7.82 \text{ for } P = 0.05$$
$$\text{and } t.\ s_d = 10.45 \text{ for } P = 0.01$$

The mean treatment effects were (see table 1) :

AA = 20.75 AG = 5.91
GG = 42.91 GA = 14.58

Although they are not orthogonal, that is to say independent, it may be useful to test the 6 possible comparisons of the simple effects.

GG/AA that is
42.91 − 20.75 = 22.16**
GG/GA that is
42.91 − 14.58 = 28.33**
AA/AG that is
20.75 − 5.91 = 14.84**
GA/AG that is
14.58 − 5.91 = 8.67*
GG/AG that is
42.91 − 5.91 = 37.00**
AA/GA that is
20.75 − 14.58 = 6.17 (N.S.)

Among these comparisons, only the first 3 are interesting ; they confirm the first 2 points of the main effects analysis.

D. Immunological relations between the two viruses

We can quantify these immunological relations and specify the confidence interval of the calculated values.

1. Unilateral relations

They can be defined for each vaccine, as the immunity variation expressed as a value of K which is produced by the heterologous test (see figure 3).

The confidence interval

$$t.\ s_d = t\sqrt{(2 \times 90)/12}$$

is that which we have already calculated (t. s_d = 0.78 after return to original data).

Allier vaccine :

r_1 = heterologous K/homologous K

or

$$\log r_1 = \log K^{he} - \log K^{ho}$$

or

− 0.41 − 1.07 = − 1.48 ± 0.78

Greece vaccine :

similarly, we have :

log. r_2 = 0.45 − 3.29 = −2.84 ± 0.78

2. Bilateral relation

This relation can be expressed as in serology

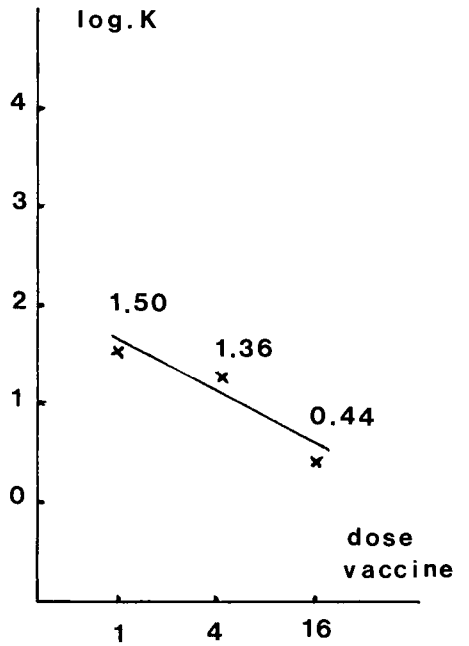

Fig. 2. — Regression line of immunitary responses according to vaccine dose.

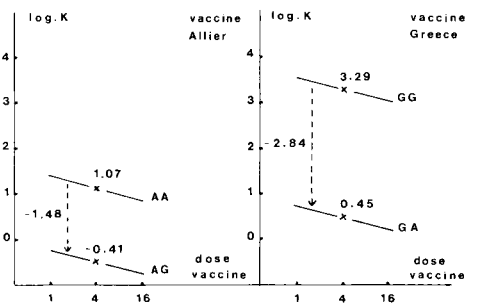

Fig. 3. — Immunological relationship between the two viruses.

by the geometrical mean of unilateral relations.

$$R = \sqrt{r_1 \times r_2}$$

or

$$\log R = 1/2 (\log r_1 + \log r_2)$$

$$\log R = [-1.48 + (-2.84)]/2 = -2.16$$

This relation represents the mean decrease in immunity of the 2 vaccines, Greece and Allier, in the presence of the heterologous virus.

The confidence interval

$$t.\ s_d = t\sqrt{(2 \times 90)/24} = 5.51$$

that is, 0.55 after returning to original data.

This bilateral relation, which measures the distance between or the immunological relationship of the 2 strains can therefore be written as :

$$\log R = -2.16 \pm 0.55$$

3. Dominance

The dominance of the A Allier virus can be expressed as the difference between the immunity falls produced by the heterologous reaction.

$$D = r_1/r_2$$

or

$$\log D = \log r_1 - \log r_2$$

$$\log D = -1.48 - (-2.84) = 1.36$$

Confidence interval

$$t.\ s_d = t\sqrt{(2 \times 2 \times 90)/12}$$

$$2.02 \times 5.47 = 11.04$$

or 1.10 after return to original data.

Finally, we have :

$$\log D = 1.36 \pm 1.10$$

As Stellman *et al.* (1972) indicate, it is preferable to express the dominance by the expression :

$$D = \sqrt{r_1/r_2}$$

We then have :

$$\log D = 0.68 \pm 0.55$$

and we notice that the confidence interval is the same as that of the R relation, that is : 0.55.

It is appropriate to note that the discovering of a dominance can be influenced by the difference in efficiency of the 2 vaccines. The possible dominance of one of the viruses can only be validly demonstrated if the 2 vaccines give rise to comparable homologous immunities, which is not the case in the chosen example.

Conclusion

A double immunological cross-test using the index K method on bovines can be considered from the statistical point of view, as a factorial experimental design. The discovery of the existence of 2 subtypes is achieved by comparing as a whole the homologous immunity values with heterologous ones.

The statistical calculations with recourse to the variance analysis, have been examplified by the virus pair, A Greece 69-A Allier. The study of the immunological cross-test carried out with these 2 virus strains, shows that it seems to involve 2 immunologically distinct subtypes, the relationship between them being :

$$-2.16 \pm 0.55 \log K$$

measured by this method.

The 2 vaccines, A Allier and A Greece used in this experiment, were of very different values. Due to this difference in efficiency, the discovery of an A Allier viral dominance is liable to caution.

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Summary

A double immunological cross-test, carried out with the index K method, is subjected to a statistical analysis by a factorial experiment. The A Greece and A Allier viruses, which have been taken as an example of the calculations procedure, seem to be 2 immunologically different subtypes.

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