The effect of solar eclipse on BT viral growth – an experimental study

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Abstract— **Complex biological processes like those involved in cell culture are subject to apparently inexplicable rate variations, generally attributed to uncontrollable microscopic fluctuations. Our State Biological Institute produces many kinds of vaccine for hundreds of millions of animals throughout India, strictly adhering to FAO guidelines, yet levels of non-biorhythm variance are often high, so production is constantly monitored. In a series of six recent experiments on two kinds of process, viral growth (4) and bacterial growth (2), we have identified a new kind of non-biorhythm variation, previously unknown to science, systematically varying with batch starting time. Cumulative statistical significance is below 10^{-22}, while the analysis of variance suggests that *it may account for up to 75% of non-biorhythm variance*. Here we report investigations of these anomalous variations on both 2012 solar eclipse days. Finally it is concluded that solar eclipses can influence starting time dependence of virus growth, and suggest that starting time effects of the eclipse result from a response of the biosphere *as a whole*, including the micro-world of cell biology. These results further validate previous conclusions that time has a heterogeneous effect on bioregulation.**

Keywords- Anomalous Variations, Biological Growth, Solar Eclipse, Starting time

I. INTRODUCTION

As in many countries, Indian agricultural policy is to provide farmers with subsidized vaccines. The Institute for Animal Health and Veterinary Biologicals (IAHVB) produces vaccines for all kinds of animals, both commercial and domestic, and even some human vaccines, against all kinds of infection. Bacterial and viral vaccine production and vaccination are routine, but unexplained variations require constant monitoring of production quantity and quality. Since scale of production makes it worth the effort to try to improve efficiency, we decided to see if there was any regularity in the variations. Inspection suggested a possible dependence of production level on starting time. All our production runs involve complex biological processes, subject to many still poorly understood levels of regulation or regulated influence, so even this seemingly unlikely possibility did not seem inherently impossible. IAHVB production strictly adheres to FAO protocols (1,2), while our assays are professionally performed. These circumstances seemed sufficient to perform cost-free experiments simply by varying start times of production runs i.e. times of inoculation of production batches – an at least harmless way of seeing if something unexpected might not be present. Thus we started to conduct the investigations reported herein.

II. SUMMARY OF EXPERIMENTS

A first, simple experiment comparing two start times for Bluetongue virus vaccine production on four different days seemed to confirm our suspicions (3) (Table 1a), so we performed another more extensive experiment on bacterial growth for Blackquarter (a bacterial disease caused by *Clostridium chauvoie*) vaccine production (4), as the process is easier to assay, and more kinds of measurement can be performed. e.g. CMI/turbidity/opacity/sporulation. Again the design was simple, start production batches at five different times on each of eight days. Results were particularly interesting since we observed significant increases in production on particular days, as well as at specific times on each day: 2-Factor ANOVAs attained significance in both factors (Table 1b).

The next experiment obtained independent confirmation of the effect. A commercial vaccine laboratory made observations of Newcastle disease virus vaccine production (4). Results of such novelty require extensive confirmation, so we continued experiments on an occasional basis. The overall statistical significance built up to 10^{-22}, a (geometric) mean of $\mathbf{p} = 3 \times 10^{-4}$ per experiment. (5) Here we present results of our two most recent experiments in detail, since they seem to have uncovered a further phenomenon, and provided a possible clue to what is going on. They came about in the following way: as experiments yielded increasingly significant statistics, the question of what might be causing the observed variations grew stronger. Some starting times definitely seemed to favor vaccine production, others to hinder it. Residing in India, we are naturally aware of traditions that speak of 'favorable' and 'unfavorable' times. One such time is during an eclipse. We decided to test production levels of a previously tested vaccine during the eclipse of 20th May, 2012, even though the penumbra never touched the Indian subcontinent at any time during the day. The eclipse's transit was from China to the South Western United States, avoiding South Asia completely.

When we found a weak effect, and that statistics required improving, we decided to conduct a repeat experiment using an almost identical protocol on the day of the second 2012 solar eclipse, 14th November. Both are reported below.

Materials and Methods

Experimental System: Bluetongue virus serotype BTV-23 infection of BHK21 (Cell line 13) cells, both from IAHVB, Bangalore.

Cell Culture Media: Eagle's dehydrated medium with L-glutamine, procured from Gibco-BRL®, USA. 1X medium prepared to manufacturer's directions in Milli-Q water; pH adjusted to 7.2 using CO_2., sterilized by membrane filtration using 0.22µm filter membranes; and stored at 4˚C.

Growth medium: prepared fresh at time of sub-culturing by adding 7% foetal bovine serum from Biological Industries®, Israel.

Maintenance medium: prepared at time of infection without serum.

Microtiter Plates: 96 well (8 row x 12 column) tissue-culture plates from Nunc®.

Micropipettes: Finn Pipettes®, 200-µl and 1000-µl single channel for addition of cells, and 300-µl multichannel for addition of virus to tissue-culture plates.

Virus dilution: 100 µl virus, serially diluted x10 from 10^{-0} to 10^{-9} in maintenance medium were added to 4 wells of successive plate columns; two wells on each plate contained virus control (neat virus+media) and cell control (cells+media).

Virus titration Co-cultivation method : 100 µl BHK21 cell suspension ($3x10^5$ cells/ml) harvested from milk dilution were placed in each well, virus addition performed; plates were then covered, sealed with cellotape, and incubated at 37°C, 5% CO_2.

Assessments: wells were observed daily for CPE (characteristic cyto pathogenic effect); the number of wells showing CPE was recorded. Final readings were taken 120 hours after infection, and $TCID_{50}$ end points calculated by the standard Reed-Muench formula.

Statistical Analysis: used SPSS16.

III. Results

On 20th May, 2102, four production batches were started at each of seven well-spaced times (Table 2a). The data yielded tantalizing results. While the ANOVA only yielded **F** = 2.52, which for df = 6/21 gave **p** = 0.0538, a 't' test comparing $TCID_{50}$ levels from the four eclipse starting times with the three non-eclipse starting times, yielded **t** = 3.13, df=26, and **p** = 0.0043.

For the second experiment, we selected three starting time slots during the eclipse, and five starting time slots after it (one additional time, Table 2b). Statistics improved: the ANOVA yielded **F** = 3.319, df = 7/24, **p** = 0.0116, while the t test on the eclipse versus non-eclipse $TCID_{50}$ values gave **t** = 3.81, df = 30, and **p** = 0.0006; both statistics attained good significance. Furthermore, since the two experiments were so similar (see Methods), data can be combined. The ANOVA then yields **F** = 2.68, df = 14/45, and **p** = 0.0062, while the t test yields **t** = 4.49 with df = 58 so that **p** < 0.00003, a really excellent result.

IV. Discussion and Conclusion

Taken all together, our results strongly suggest that we are looking at some real 'starting time' effect in biological processes. In particular, starting times during the occurrence of a solar eclipse have different effects on Bluetongue virus growth from starting times on the same day after the eclipse has finished. In this context, 't' tests found no significant difference between non-eclipse time slots (**t** = 0.81, df = 30, **p** = 0.42), but a significant difference between the two eclipse time periods, (t = 2.36, df = 26, p = 0.026). The two eclipses seemed to exert different levels of effect. What is going on?

Complex biological processes are subject to variation, as is well-illustrated by $TCID_{50}$ values. Rows of TC plate wells do not behave uniformly, in each row different numbers show CPE. The test incorporates variability of viral growth, the Reed-Muench $TCID_{50}$ formula expresses it. Such variations in complex biological processes have always been thought to depend on such things as chemical and temperature fluctuations, and possibly originate in genetic variation. Our experiments are new in two respects: (1) identification of batch starting time as an independent variable potentially accounting for substantial percentages of observed variance; and now (2) *identification of eclipse times as supportive to virus production*. Is time exerting a heterogeneous influence on complex biological processes? If so, accurate predictions of its effect would improve vaccine production.

What of our finding on eclipse times? Observations of eclipses have reported changes on many different levels, ionospheric (6), atmospheric (7), gravitational waves (8), meteorological (9), chemical (10), hydrological (11), and record consequent trauma to many life forms such as birds (12), fish (13), rodents (14), and primates (15). Effects on microbes have also been noted (16, 17). Most are local effects, few relate to regions far from totality. However, if their total influence affects the *biosphere as a whole*, a possible causal chain for our observations would be:

Eclipse ➔ Trauma to local fauna ➔ Global trauma to biosphere ➔ Influence on distant life forms

This kind of response pattern might be the beginning of an explanation for our anomalous observations.

What might cause the observed variations? Purely chemical processes will not exhibit them; we suggest that they may originate in biological complexity. What aspect of complexity biology might be involved? This question obviously requires careful consideration. In our opinion complexity may be the simplest and most promising place to start searching for mechanisms.

Finally it is concluded that the results of present experimental investigation are in excellent correlation with the concept of time &space as enunciated in the ancient Indian scientific literature the scope of this paper can further be extended by conducting various combination of the experiment with variable parameter with time &space this pioneering work is unique of its kind in the world and may represent a new dimension in microbiology. 'Starting time effects' require further investigation; one independent verification is not enough.s.

TABLE 1a: Comparison of Bluetongue Virus Infection of BHK21 (Cell Line 13) Cells for Two Starting Times

TIME ⇨ DAY ⇩	Cultivation METHOD	TIME A $TCID_{50}$	TIME B $TCID_{50}$	TIME B minus TIME A
Day 1 25.08.11	Monolayer	5.76	6.31	+ 0.55
Day 1 25.08.11	Cocultivation	5.36	6.24	+0.88
Day 2 29.08.11	Monolayer	4.75	5.25	+ 0.50
Day 2 29.08.11	Cocultivation	4.63	4.75	+ 0.12
Day 3 02.09.11	Monolayer	5	6	+ 1.00
Day 3 02.09.11	Cocultivation	4	5	+ 1.00
Day 4 06.09.11	Monolayer	5.18	7.66	+ 2.48
Day 4 06.09.11	Cocultivation	5	7.24	+ 2.24
	Mean	4.96	6.06	1.10
	St Deviation	0.525	1.038	0.836
	t Value	Unpaired / Paired	2.67	3.72
	p Value	p =	0.0181	0.0074

Table 1a presents $TCID_{50}$ values of Bluetongue virus concentration obtained in two related assays on four different days started during two different time slots, for which no difference could be theoretically expected when averaged over the precisely maintained 120 hour incubation period. Consistency of differences between Time A and Time B makes a sign test a simple means to establish that differences between the two columns are significant p = 0.00781. The consistency of observed differences between cocult and monolayer cultivation methods indicates that errors in measure result in standard deviations considerably smaller than the mean difference of 0.46. When a paired t test is performed on the differences column, we obtain t = 3.72, for which 7 degrees of freedom yields, p = 0.0074. Subjecting this dataset, with monolayer/cocult differences partialled out, to a 2-Factor ANOVA yields F=5.60 for the days, for which we obtain p = 0.0140 (df = 3/11). Variations of starting time effects with different days appear to have their own significance – for more on this, see Table 1b.

TABLE 1b: Anomalous Dependence of Overall Growth of *Clostridium Chauvoei* on Starting Times during BQ Vaccine Production (Spectrophotometric measures of Nephlometric Turbidity Units)

TIME ⇨ DATE ⇩	TIME A	TIME B	TIME C	TIME D	TIME E
12.10.11	155	420	246	179	190
13.10.11	295	300	314	434	412
17.10.11	123	380	228	146	175
18.10.11	224	370	285	180	184
21.10.11	293	422	285	176	305
22.10.11	133	400	232	224	128
26.10.11	138	327	265	163	155
28.10.11	160	332	260	165	224
MEAN	198.1	368.9	264.4	208.4	221.6
SEM	25.1	16.0	10.4	33.2	33.0

Table 1b presents Nephlometric Turbidity Unit (NTU) measurements of *Clostridium Chauvoei* after 48 hours growth in standard growth medium. The different columns, A, B, C, D, and E were started at given times on each day. The various starting times show consistent, large variations in bacteria production for vaccine, varying from 123 to 434 – a factor of 3.5. Time B tended to yield the largest production each day, while Time C tended to be second largest. Similarly starting times on 13th October, tended to be the largest in each column, and those on 21st October second largest. Not surprisingly, a 2-Factor ANOVA yields F = 12.17 for the columns (df = 4/28) i.e. starting times each day, and F = 3.58 for the rows (df = 7/28) i.e. different days, suggesting that starting time influences may vary with time of day, *and* that they may also vary in their effects quite abruptly from day to day. Corresponding null hypothesis p values are 0.0071 (days) and approximately 3 x 10^{-6} (times of day). Datasets for CMI (continuous values), opacity (ordinal, 4-10), and sporulation quality (ordinal, 1-3) were also obtained with similar ANOVA F values and P values.

TABLE 2a: Data Table from Eclipse of 20th May, 2012

TIMES ⇨ BATCH ⇩	A	B	C	D	E	F	G
1	7.5	7.23	7.5	7.66	7.78	6.45	7.34
2	6.55	6.51	7.51	7.77	6.50	6.50	6.78
3	7.23	7.50	7.34	7.78	7.34	6.34	6.34
4	7.51	7.34	7.51	7.33	6.66	7.55	6.45
Mean	7.20	7.15	7.46	7.63	7.07	6.71	6.73
StDev	0.45	0.44	0.08	0.21	0.60	0.56	0.45

: Table 2a presents TCID50 values sets of 4 batches started during successive time slots on 20th May, 2012, the day of the year's first total solar eclipse. Each set ofl four batches was started within the same time slot, and their means and standard deviations are given. The question of whether different starting times result in observably different results is answered by performing an ANOVA, which yielded the p value, p = 0.0538, suggesting that, some effect might possibly be there, though more data s needed. The question of whether eclipse times (Times A, B, C and D) produced different results from non-eclipse times (Times E, F and G) is answered by performing a 't' test between the two blocks of data. The result is **t** = 3.13, which, for df = 26, yields **p** = 0.0043, seeming to suggest a probable, 'Yes'. This encouraging result led to our performing the second experiment, the data for which is given in Table 2b.

TABLE 2b: Table of $TCID_{50}$ Data from the Eclipse of 14th November, 2012

TIMES ⇨ BATCH ⇩	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**P**
1	7.66	7.33	8.77	6.78	6.50	6.30	7.23	7.50
2	7.23	7.50	7.55	7.50	6.66	7.23	7.66	6.44
3	8.77	7.23	7.78	6.34	7.23	6.51	8.33	6.51
4	7.78	8.33	7.34	7.54	7.50	6.66	6.78	6.78
Mean	**7.86**	**7.60**	**7.86**	**7.04**	**6.97**	**6.68**	**7.50**	**6.81**
StDev	**0.65**	**0.50**	**0.63**	**0.58**	**0.47**	**0.40**	**0.66**	**0.48**

Table 2b presents $TCID_{50}$ values of sets of 4 batches of BT virus vaccine started during successive time slots on 14th November, the day of 2012's second total solar eclipse. Here, time slots H, I and J were during the eclipse, while the rest, K to P, were after it had finished in South America. All four batches were started within the given time slots. In this case, the ANOVA yielded **F** = 3.319 with df = 724, giving **p** = 0.0116, a reasonable indication that effects are probably present. The 't' test between the blocks of data gives **t** = 3.81, which, for df = 30, yields **p** = 0.0006, indicating that 'eclipse' and 'non-eclipse' time slots do, most probably, produce different starting time effects on BTV growth in BHK21 (Cell line 13) cells. **Added Note**: clearly, Tables 2a and 2b are of exactly the same form, and concern the same phenomenon, so it seems reasonable to repeat the 'F' and 't' tests on the combined data: **F** = 2.68, which for df = 14/45, gives **p** = 0.0062, an estimated expectation of less than one part in one-fifty that the null hypothesis is correct, while **t** = 4.49, meaning that, for 58 degrees of freedom, **p** < 3 x 10^{-5}. (In contrast, taking the p values of Tables 2a and 2b as independent yields a combined p value of **p** = 6 x 43 x 10^{-8} = 2.5 x 10^{-6}). We feel that even the conservative estimate of **p** makes it worth taking these rather unexpected results quite seriously. Finally, it is also of interest that a 't' test between the two sets of eclipse data, columns A-D, vs. columns H-J, yields **p** = 0.026. Might this suggestive result be associated with the first eclipse being only an annular eclipse, while the second was total?

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