THE GROWTH OF PLEUROPNEUMONIA-LIKE ORGANISMS OF HUMAN ORIGIN: CULTIVATION IN THE DEVELOPING CHICK EMBRYO AND AN IN VITRO GROWTH CYCLE

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Some strains of pleuropneumonia-like organisms (PPLO) of human origin grow quite sparingly *in vitro*; therefore, attempts were made to determine if these organisms could be sustained better in the developing chick embryo. A semiquantitative comparison of growth on artificial media and in the chick embryo is presented. As pleuropneumonia-like organisms are frequent inhabitants of the genitourinary tract and have often been found to be associated with pathological processes, as summarized by Morton *et al.* (1952), attempts were made to ascertain if any pathological involvement or increase in mortality could be noticed in the infected chick embryo.

The only report of the cultivation of pleuropneumonia-like organisms from man has been a recent report by Paine *et al.* (1950). These workers describe the isolation of a strain of pleuropneumonia-like organisms from the cerebrospinal fluid of a 20 year old male who was suffering from meningitis. This strain was cultured subsequently in the yolk sac.

In the studies of the growth of pleuropneumonia-like organisms in the developing chick embryo, as in other investigations, we have had the need of quantitative information on the growth of these organisms in order to be able to choose correct and reproducible inocula. The only report dealing with this problem is a study by Holmes and Pirie (1936). These investigators estimated the growth of the pleuropneumonia organisms of cattle by plotting glucose utilization and reduction of methylene blue in the presence of sodium lactate in regard to the age of the cultures. It was found that a rapid increase of the enzymatic functions occurred for two days which leveled off at about three days.

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In this study we present the growth cycle of pleuropneumonia-like organisms of human derivation calculated by turbidimetric and viable count techniques.

MATERIALS AND METHODS

Cultures. All strains of pleuropneumonia-like organisms used in these studies are isolates from the human genitourinary tract. Strain Campo was received from L. Dienes, Massachusetts General Hospital. Strain 07 was received from I. G. Schaub, Johns Hopkins University. Both strains are isolates from the urethra. A strain from the prostate, strain 60; two strains from the cervix, strains 39 and 110; and one strain from the male urethra, strain 48, were isolated by Dr. Paul F. Smith. All six strains were employed for attempted cultivation in the developing chick embryo, but only the two strains, Campo and 07, which grow well on artificial medium were employed for the *in vitro* studies.

Media. Bacto-heart infusion broth and Bacto "PPLO-agar" (Morton *et al.*, 1951) both enriched with "Bacto-PPLO" bovine serum fraction A (Smith and Morton, 1951) were utilized as artificial media.

Nine to eleven day old fertilized Leghorn chick eggs were candled and checked for viability. Allantoic sac inoculations were made employing essentially the methods described by Beveridge and Burnet (1946). A small hole was drilled at the margin of the air sac, and 0.2 ml of the three day old broth cultures of the strains was inoculated intraallantoically. Tuberculin syringes and 26 gauge hypodermic needles were routinely employed. The openings were sealed with scotch tape and incubation carried out for three days at 37 C. At the end of this period the allantoic fluid was harvested by conventional methods. Two-tenths ml of this freshly harvested allantoic

fluid was introduced then in the same manner into a new group of normal chick embryos. At the same time loopfuls of the harvest were streaked onto the agar medium. After three days of incubation at 37 C the plates were examined for the characteristic colonies with the microscope employing a magnification of 100 ×. These steps are necessary to determine whether growth had taken place in the allantoic fluid, as growth of pleuropneumonia-like organisms cannot be determined by staining procedures. As there is a report in the literature of an isolation of pleuropneumonia-like organisms from an apparently normal chick embryo (Van Herick and Eaton, 1945), one dozen randomly chosen chick embryos were checked for the presence of pleuropneumonia-like organisms. In this case as in others, none of the uninoculated embryos yielded pleuropneumonia-like organisms.

Allantoic fluid freshly harvested was dispensed into sterile test tubes. These were inoculated with a loopful of the strains. After three days of incubation at 37 C, loopful transfers were made into freshly harvested allantoic fluid. The plating method described above was utilized to ascertain if growth had occurred. The experiment was terminated after six consecutive transfers.

Turbidimetric determinations. Chemically cleaned, sterile Klett test tubes were filled with 5 ml of the enriched "Bacto-PPLO" broth. One loopful of 4 day old culture of the strains was utilized as an inoculum. The tubes were incubated at 37 C. Determinations were made every 8 hours with a Klett-Summerson photoelectric colorimeter employing a no. 420 filter. Figure 1 is a reproduction of a typical result.

Viability determinations. One drop of a 4 day old culture of the strains was inoculated into small neck flasks containing 50 ml of enriched "Bacto-PPLO" broth. This was incubated at 37 C. At periodic 8 hour intervals, 0.5 ml of the growing culture was removed and serially diluted with 4.5 ml of the enriched broth medium. Individual sterile one ml pipettes were used for each transfer in the dilution series, and each tube was mixed 5 times to insure complete distribution. These tubes were incubated then at 37 C for 3 and 5 days, at which times the different dilutions were checked for visible turbidity and plated onto agar medium. After 3 days of incubation these plates were inspected microscopically for the typical colonies produced by pleuropneumonia-like organisms. The highest dilution of the broth tubes manifesting active proliferation was taken as an indication of the population increase in the primary flask cultures. Figure 2 incorporates the results of these experiments.

Plate count determinations. Colonies of pleuropneumonia-like organisms can only be recognized with the aid of a microscope employing a magnification of 100 ×. Deep colonies formed by a pour plate method would not be readily recognizable; therefore, a volume to area surface plating method was utilized. One-half ml of a 1:10 dilution of a 4 day old culture of the strains was used as an inoculum. As before, 50 ml volumes of broth were distributed into small neck flasks, inoculated, and incubated at 37 C. Aliquot samples taken at intervals of 4 to 8 hours were diluted in a serial tenfold fashion, in the manner described above. One-tenth ml samples of the various dilutions then were plated onto

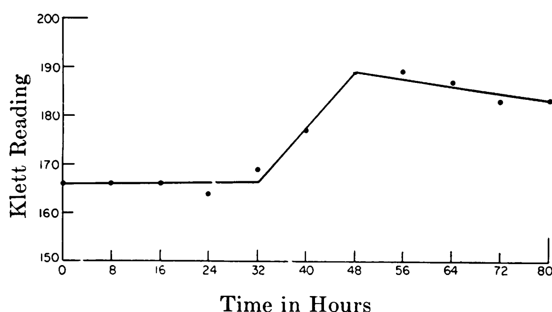

Figure 1. Turbidimetric changes in a culture of pleuropneumonia-like organisms.

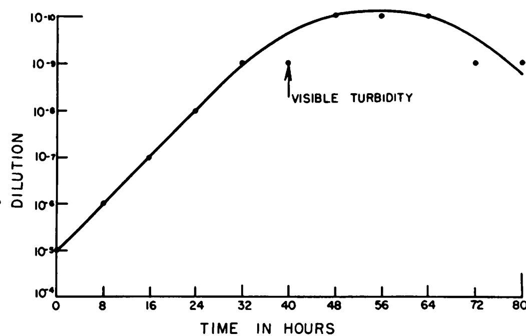

Figure 2. Viability of pleuropneumonia-like organisms during the growth cycle as determined by the manifestations of growth in broth tubes serially diluted with aliquot samples from the growth flask.

agar medium contained in two inch petri dishes and evenly spread with sterile bent glass rods.

After 3 days of incubation at 37 C, plates showing an average count of 10 colonies per microscopic field were chosen for readings. The plates were scanned in a regularly repetitive pattern. This was accomplished with the aid of a small wooden frame which was fitted onto a mechanical stage. The small petri plates were inserted into the wooden frame and then could be manipulated as easily as a glass slide.

The area of the two inch petri dishes and the area of the low power, 100 ×, field of an American Optical Company microscope were determined and found to be in a 1:988 ratio. For calculation this was considered as a 1:1,000 ratio. The plating inoculum was 0.1 ml. The viable organisms per milliliter could be calculated then by the use of a factor of 1:10,000, the average number of colonies of 100 fields counted, and the designated dilution factor of the sample. Eight replicate experiments demonstrated no significant variation in the counts by this procedure. The results were plotted on a logarithmic scale. A typical curve is reproduced in figure 3.

RESULTS AND DISCUSSION

The results of the growth of pleuropneumonia-like organisms in the developing chick embryo are summarized in table 1. The strains which grew well on artificial media grew well in the chick embryo. The strains which grew poorly *in vitro* did not reproduce any better *in vivo*. Strain 07 was grown consecutively for 13 trans-

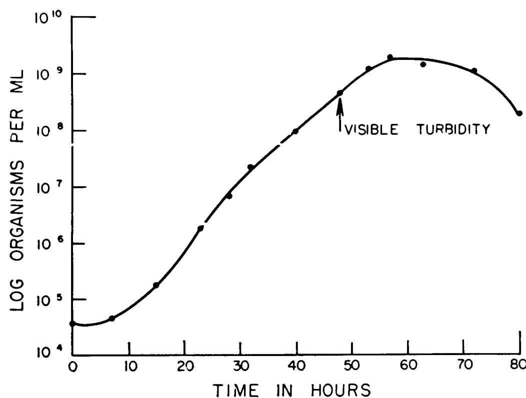

Figure 3. Viability of pleuropneumonia-like organisms during the growth cycle determined by a colony count method.

TABLE 1

Comparison of growth of pleuropneumonia-like organisms on artificial medium and in the chick embryo

STRAINS	ARTIFICIAL MEDIUM	CHICK EMBRYO	NUMBER OF TRANSFERS
07	++++*	++++*	13**
CAMPO	++++	++++	6
60	+++	+++	6
39	+++	+++	6
110	++	++	2
48	+	–	0

* ESTIMATION OF GROWTH. ++++, MAXIMUM GROWTH; +, SCANT GROWTH; –, NO GROWTH

** NUMBER OF CONSECUTIVE TRANSFERS THAT THE PPLO WERE CARRIED IN CHICK EMBRYOS.

fers. Strains Campo, 60, and 39 were grown for 6 consecutive transfers, and the experiment was terminated at that time. We were unable to recover viable cells of strain 110 from the third transfer. Strain 48 which grows very poorly and erratically in artificial liquid medium did not grow in the chick embryo. No change in the colonial morphology was noted after the chick embryo passages.

All the strains which grew in the chick embryo proved capable of multiplication in the *in vitro* allantoic fluid as well. The strains grew in the same manner as they did in the chick embryo.

The effect of growth of pleuropneumonia-like organisms in the chick embryo was determined by candling the embryos. The chick embryos retained their viability after three days of growth of the pleuropneumonia-like organisms. In order to study the effect of proliferating pleuropneumonia-like organisms on the development and normal hatching of the chick embryos, 80 inoculated embryos were allowed to undergo their normal 21 day hatching period. The chicks were hatched in an Oakes no. 887 electric hatcher. The chick eggs were cooled and turned twice a day in accordance with the prescribed hatching procedure. The inoculation procedure was the same as described earlier. The strains of pleuropneumonia-like organisms used were Campo, 07, and 39. For controls, sterile liquid medium was introduced into 50 chick embryos. No significant differences in the percentage of mortality and no

obvious changes in the state of health of the newly hatched chicks were observable. Sporadic checks proved that pleuropneumonia-like organisms could be isolated from inoculated unhatched chicks during both the early and late stages of development. There was no change in the color or consistency of the allantoic fluid.

Most of the strains grew equally well in both the chick embryo and in the artificial medium, indicating that there is no deficiency in the medium which the chick embryo system could supply. With the two more fastidious strains, growth was better on artificial medium than in the developing chick embryo. Strain differences may be playing an important role. As pleuropneumonia-like organisms can be grown in an animal system, such as the chick embryo, which has been shown to be naturally free of these organisms, it may be possible to investigate the matter of cytoplasmic inclusion bodies. Inclusion bodies have been shown to occur with infection of animals with the pleuropneumonia group (Nelson, 1935, 1937, 1940) and have been reported in cases of nonspecific urethritis of humans when infection with pleuropneumonia-like organisms was proven or suspected (Findlay and Willcox, 1951; Borrel, 1952).

We have made some studies on this problem by cultivating our strains for 3 days on the dropped chorioallantoic membrane of 4, 9, and 13 day old chick embryos. Impression smears were made of the isolated and washed membranes. The preparations were fixed with 10 per cent formalin solution and Carnoy's solution, containing 6 parts ethyl alcohol, 3 parts chloroform, and 1 part glacial acetic acid. Staining procedures were attempted by the Gram, Giemsa, and Wayson methods. All of these studies have yielded inconclusive results. Our inability to notice any obvious pathological involvement in the chick embryo or hatched chicks may be due to a rigid species and organ specificity of the organisms (Sabin, 1952; Smith *et al.*, 1952), or due to employing strains which have been on artificial medium for many generations. Pleuropneumonia-like organisms have been found often to be infectious only for the species from which they were isolated originally. The part that pleuropneumonia-like organisms play in the production of nonspecific urethritis is still uncertain. It is hoped that the chick embryo may find use as an experimental tool in clarifying this problem.

The turbidimetric determinations show that no increase in turbidity occurs for about 32 hours (figure 1). A fairly rapid increase in turbidity occurs for the next 16 hours. From 48 to 80 hours there is again a fairly constant state. The obvious conclusion drawn from these results that the major part of the reproduction was taking place at the time of increasing turbidity was proven incorrect by the viable count experiments. The amount of turbidity one notes in a fully grown culture is very faint. It is not easily noticeable upon visual examination and needs comparison with an uninoculated tube to be discernible. The reason for this is the minute size of these organisms, estimated by gradocol membrane filtration methods to range from 125 to 500 millimicrons (Laidlaw and Elford, 1936). This late increase of turbidity has been noticed routinely with all of our culturing of this organism.

That visible and measurable turbidity becomes evident only at a late stage of development and at a time when the organisms have reached nearly maximal viability may be explained by one or all of the following: (1) Because of the small size of these organisms, the number of organisms required to produce a measurable turbidity is not reached until this late stage in the growth cycle, (2) an increase in density is occurring in the organisms at that time, (3) an increase in the size of the organisms with the production of "large bodies" occurs during that period. In this connection, the production of these forms as reported by Klieneberger and Smiles (1942) and Dienes (1945) should be noted.

From inspection of the curves plotted by the two methods of viability determination (figures 2 and 3), it becomes evident that the organisms undergo a normal bacterial type of growth cycle. There is evidence for a lag period of about 8 hours' duration under the above conditions of cultivation. From this time until 53 hours there is a logarithmic increase of the numbers of viable organisms. This is followed by a very short period of growth in the stationary phase which only lasts about 19 hours. From then on the count of the viable organisms declines. This occurs in a gradual manner. The culture usually becomes sterile in about two weeks.

The morphology and reproduction of the organisms in the pleuropneumonia group have

been exceedingly disputed topics. The electron microscopic studies of Smith *et al.* (1948) give some evidence for binary fission. Recent studies with the electron microscope by Tulasne and Bringmann (1952) cite further evidence for reproduction by binary fission. The production of "large bodies" by an increase in the size of the elementary forms and their subsequent disintegration has been interpreted by Klieneberger and Smiles (1942) and Dienes (1945) as being a complex reproductive phase of these organisms. That the swelling of the rod and coccal forms to produce the large bodies is essentially degenerative in nature has been postulated by Orskov (1927). Freundt (1952) investigating the nature of the "large bodies" came to the same conclusion. He noticed that the "large bodies" did not appear until the later stages of growth and correlated them with a decrease in the viability of the culture. He presents evidence in support of the "large bodies" being formed by plasmoptysis caused by an increase in permeability of the cells due to a lowering of pH within the culture. Knaysi (1951) also has expressed the opinion that the swollen cells of the pleuropneumonia organisms are formed by action of mild plasmoptysis. From our results it appears that if the "large bodies" arise in a phase-like manner, they are not correlated with a reproductive function as there are not stepwise deviations in the logarithmic phase (see figure 3). If the large bodies appear at a late stage as has been reported by Freundt, this would also invalidate a reproductive function of the swollen forms as no marked increase in the viability count occurred when turbidity appeared. That the large bodies produce a minor part of the reproductive elements which are masked then by binary fission certainly cannot be discounted and needs further investigation in order to be elucidated.

In order to calculate the generation time of these organisms, binary fission was assumed to be the sole method of reproduction. The average generation time during the logarithmic phase of growth was determined to be 3.27 hours. Using the T-test, the 95 per cent confidence intervals were found to be 3.27 $\pm$ 0.78 hours. The maximum number of viable organisms in the stationary phase was determined to be approximately 1.5×10^9 per ml.

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SUMMARY

Five strains of pleuropneumonia-like organisms isolated from the human genitourinary tract were cultivated intraallantoically in the developing chick embryo. A sixth strain failed to grow. Harvested allantoic fluid also favored multiplication of all of the strains of the organisms which grew in the chick embryos. For the majority of the strains there was no difference of the growth of these organisms in the chick embryo and in an artificial medium. For the more fastidious strains multiplication was better on artificial medium than in the developing chick embryo. Studies on the pathological manifestations of these organisms multiplying in the developing chick embryo have shown no significant degree of mortality or obvious involvements of the hatched chicks. In this respect the strains studied differ from pleuropneumonia organisms of cattle.

A growth cycle of pleuropneumonia-like organisms of human origin is presented. On the basis of turbidimetric and viable count methods, it is shown that these organisms follow a normal bacterial growth cycle. The reproductive patterns of pleuropneumonia-like organisms are discussed. Assuming that binary fission is the major mode of reproduction, the generation time of these organisms during the logarithmic phase was calculated to be 3.27 $\pm$ 0.78 hours.

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