Veterinary Serum and Vaccine Research Institute,
Abbassia, Cairo, Egypt.
Director Prof. Dr. N.I. Hassan.

STUDIES ON THE PROPERTIES OF DUCK VIRUS HEPATITIS VACCINE

(With 7 Tables and 3 Figures)

By

ELHAM A. EL-EBIARY; NADIA M. HASSAN; S.W. AHMED; ENSAF M.H. KASHABA; FEKRIA EL-BORDINI; SALWA EL-ASSILY and AFAF ABD EL-HADY

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دراسات على خصائص فيروس الإلتهاب الكبدي في البـــط

الهام الأبيـــاري ، نادية حســـن ، صلاح أحمـــد ، أنصاف خشبـــــة
فكرية البرديني ، سلوي الأصيلي ، عفاف عبد الهـــادي

تتلخص نتائج التجارب التي أجريت لدراسة خصائص الفيروس المسبب لمـــرض الإلتهاب الكبدي عند البط ومحاولات تحضير دفعة لقاح في المعمل عن التالي : كانـــت أعلي نسبة عدوي في أجنة البيض بالحقن عن طريق التجويف الألنتوني وصلت الـــي ١٠٠٪ فالغشاء اللفائفي ٩٠٪ وأخيرا تجويف الصفار ٧٥٪ ـ ووجد أن أفضل طرق حقـــن الفيروس هي الحقن عن طريق الغشاء اللفائفي حيث أعطي أعلي قوة عيارية وصلـــت إلي ^{٦}ر^{٨}١٠ / ١ سم ، بينما وصلت إلي ^{٣}ر^{٨}١٠ / ١ سم ، ^{٧}ر^{٨}١٠ / ١ سم للتجويـــف الالنتوني وتجويف الصفار علي التوالي ـ ووجد أن أفضل ساعة لتجميع فيروس الالتهـــاب الكبالكبدي للبط هو ٧٢ ساعة بعد الحقن حيث وجد أن القوة العيارية بلغت $^{(ر٩}$١٠ / ١ سم وذلك بالنسبة للحقن عن طريق التجويف الانتوني ، ^{٥}ر^{٨}١٠ / ١ سم للحقن من طريـق الغشاء اللفائفي ـ ووجد أن أفضل مكان لتكاثر الفيروس هو أنسجة الجنين ككل حيـث وجد أن القوة العيارية كانت ١٠ / ١ سم في حين كانت ^{٦}ر^{٦}١٠ / ١ سم ، ^{٥}ر^{٦}١٠ / ١سم في كل من الغشاء اللفائفي ، والتجويف الالنتوني علي التوالي ـ وأسفرت نتائج تحمـــل الفيروس لدرجات الحرارة عن فقد الفيروس قوته العيارية عند ١٠م في حين كانـــت ٨ر ، ٨ر ، ٧ر١ ، ٨ره لوغاريتم عند تركها مدة ١ ساعة عند ٥٢م ، ٣٧م ، ٤٠م ، ٥٦م على التوالي ـ كانت نتائج إختبار صلاحية اللقاح الحي لفيروس الالتهاب الكبدي عند البـط أن معدل فقد الفيروس عيارية وصل ٢ لوغاريتم بعد ٩ شهور تخزين في الثلاجة ٤م ، بعد ٧ شهور من الحفظ في درجة حرارة الغرفة إنخفضت القوة العيارية للفيروس ١ر٦ لوغاريتم عن القوة العيارية الأصلية في حين عند ما تم حفظ اللقاح عند ـ ٢٠ م لأكثر من عام لـــم تظهر أي شواهد ملحوظة على فقد الفيروس لقوته العيارية .

SUMMARY

The results of experiments for studying the properties of live virus that causing duck hepatitis virus (DHV) disease in duckling and trial to prepare a laboratory batch of the vaccine are summarized in the following: The higher percentage ofthe pathogenicity to the embryo was 100%

when the virus was inoculated via allantoic cavity (A/C) and 9-%, 75% for chorio-allantoic membrane (CAM) and yolk sac (Y/C) respectively. The best route of inoculation of the virus was on CAM which gave the highest titer ($10^{8.6}$/ml), while the titer was $10^{8.3}$/ml and $10^{7.8}$/ml for A/C and yolk sac respectively. The best hour for harvestation of DHV was 72 hours post inoculation (PI) since the titer was $10^{9.1}$/ml for A/C and $10^{8.5}$/ml for CAM. The best site for virus multiplication is the whole embryo tissues as the titer obtained was (10^{9}/ml), while it was $10^{6.6}$/ml, $10^{6.5}$/ml in both CAM and ammnio-allantoic fluid (AAF) respectively. The results of thermostability revealed that the loss in virus titer was 0 at +10°V while it was 0.8, 0.8, 1.7 and 5.8 $\log_{10}$ when held for 1 hour at 25°C, 37°C, 40°C and 56°C respectively. The results of keeping quality of the freez-dried live (DHV) vaccine showed that the average loss of virus titer was 2 log after 9 months storage at the refrigerator temperature (+4°C). After 7 months storage at room temperture there was 6.1 log drop in the virus titer than the original virus, while when the virus was kept at the deep freezer (-20°C) for more than 1 year no significance loss in the virus titer was detected.

INTRODUCTION

Duck hepatitis virus (DHV) is a highly contagious acute viral disease of young ducklings. Infection place during the first 2-3 weeks of life causes high mortality rate (ASPLIN & MCLOUCHIAN, 1954; SHALABY, 1972 and SOKKAR et al., 1975). The most practical means of controlling infectious hepatitis in duckling is through vaccination of breeding stock to produce passive parentral immunity in their progoney (ASPLIN, 1958) and actively immunization of susceptible day-old duckling with a pathogenic DHV modified vaccine (ASPLIN, 1961 and ZUBTSOVA, 1971). This finding pointed out the importance of producing a live modified virus vaccine for duck heptatitis virus disease.

The aim of the present work is to study the properties of vaccinal strain of DHV including the following:

1- The effect of different route for vaccine production, the best of harvest of infected embryo and the target organ that give us high titer for virus reisolation.
2- Study the physical properties as determination of its heat stability at various temperature using the fluid vaccine with a holding period of one hour.
3- Study the keeping quality as freeze-dried live virus with 10% skimed milk as stabilizer at different temperature degrees [room temperature, refrigerator (+4°C) and deep freezer (-20°C)]. The importance lies in the fact that changes in temperature consequently affect the quality of produced vaccine.

DUCK VIRUS HEPATITIS VACCINE

MATERIAL and METHODS

1- Embryonated chicken eggs (ECE):

6-11-day old ECE were obtained from General Poultry company, used for virus propagation and titration.

2- Vaccinal strain of DHV:

A modified egg adapted mild living virus derived from viral hepatitis in the goos-lings, cultivated on chicken embryo (SPF). The virus was obtained from (IFFA MERIEUS), and has been propagated twice in ECE.

3- Preparation of DHV egg-propagated vaccine:

The technique of TOTH (1969) was used, where the AAF, as well as the CAM and the whole embryo without head and feet of inoculated eggs were asceptically harvested after refrigeration for few hours. The tissues were first homogenized and then mixed with AAF, adding (500 I.U. penicillin and 2 mg strep./ml) and cleanified by centrifugation at 3000 r.p.m. for 15 minutes. The supernatant fluid plus 10% skimmed milk as stabilizer was dispensed into ampouls, each contain 1 ml and lyophilized. The EID_{50} of the virus after dehydration was estimated as $10^{8.3}$/ml. It was kept at -20°C until used. The virus is regularly used as seed virus for different purpose in this study.

4- Virus titration:

It was carried out according to ANON (1971). The EID_{50} was calculated by the method of REED and MUENCH (1938).

EXPERIMENTS

Exp. 1: Trials for determination of the pathogenicity of DHV to ECE via the different routes of inoculation, A/C, CAM and Y/S with the virus diluted as 10^{-3} EID_{50}. Sixty ECE were used in this experiment. The eggs were divided into 3 equal groups, the first group was inoculated with 0.1 ml. of DHV via Y/S using 6-day-old ECE. The second group was inoculated via A/C on the 10th day-old with 0.1 ml/egg. Theeggs of the 3rd group were inoculated on the CAM using 11 day-old ECE and 0.2ml/egg. All eggs were candled daily for 7 days P.I. The percentage of +ve were calculated according to the specific lesions for DHV.

Exp. II: Effect of different inoculation routes on virus infectivity titer (EID_{50}):

Three equal groups of 25 eggs each were used in this experiment. First group was inoculated via A/C using 10 days old ECE. The eggs in the second group were inoculated with the virus on the CAM of 11th day old ECE. The third one was 6 day old ECE and was inoculated via Y/S. The EID_{50} was calculated according to REED and MUENCH (1938).

Exp. III: Trial for determination of the target organ for virus reisolation:

Thirty embryonated chicken eggs were used in this experiment. The embryonated eggs were inoculated via A/C with 0.1 ml/egg of DHV vaccinal strain diluted as 10^{-3}.

The inoculated eggs were candled daily. The AAF, CAM and the whole embryos were harvested separately. The CAMs and embryos were washed by shaking in sterile saline solution to make them free from any virus contained in AAF. The separately harvested parts of eggs were pooled. The CAMs and embryos were ground in blender with sterile salien solution (containing 500 I.U. penicillin and 2 mg strep./ml) to make 50% suspension. these suspensions were centrifuged for 15 minutes at 3000 r.p.m. and the supernatant fluid was used as the material for the titration. The allantoic fluid and these suspensions were kept at -20°C until the time of titration.

Exp. IV: Determination of the best day of harvest for DHV:

Two equal groups of 20 each, 9-10 day old ECE were used in this experiment, both groups were inoculated with 10^{-3} diluted strain of DHV via AC & CAM routes respectively. At 48, 72, 96 and 120 hours post inoculation 5 eggs were chilled at +4°C and from which the whole embryo, CAM and AAF were collected homogenized and titrated to determine the EID_{50} of virus at each previous time.

Exp. V: Stability determination of the virus fluid samples at various temperature degrees:

One ml amount of undiluted harvested fluid of the DHV vaccinal strain was distributed into serial tubes sealed, and maintained in water bathes at +10°C, 25°C, 37°C and 56°C for 1 hour. After removal of the tubes, they were kept at -20°C till the time of titration. The EID_{50} was determined after REED and MUENCH (1938).

Exp. VI: Determination of the keeping quality of the freez-dried vaccine:

DHV antigen plus 10% skimed milk as preservative was freez-dried and examined for its keeping quality at different temperature degrees. The vials were kept at room temperature, refrigerator +4°C and deep freezer (-20°C). Sample was titrated weekly from vials kept at room temperature for 7 months, on the other hand, vials from +4°C and -20°C were titrated monthly for 9 and more than 12 months.

RESULTS

Table 1: Showed the results of the pathogenicity of the vaccinal strain of the DHV when inoculated via different routes A/C, CAM and Y/S. It was found that the percentage of thepathogenicity of the vaccinal strain of DHV was 100% deaths in embryos when the virus inoculated via A/C and 90% and 75% when inoculated via CAM and Y/S respectively. It was found that the peak of mortality lies at 72 hours pot inoculation. The embryos that died between 2-4 days P.I. showed stunted growth, skin haemorrhage and reddening all over the body characteristically at the base of the toe nail, also the body had extensive oedema in the abdominal cavity, thighs and arround the head. The infected embryos that survive until 5-6 days P.I. showed localized lesions in the liver where it had greenish discolouration differ from pin point size to area affected the entire liver, the affected embryos appear stunted in growth pale and the egg yolk look greenish discolouration Fig. (2,3).

Table 2: Showed the best route of inoculation on virus titer. From the result it was found that there was no significance difference between A/C and CAM route

of inoculation on the virus titer as the EID_{50}/ml was $10^{8.3}$, $10^{8.6}$ when the DHV vaccinal strain was inoculated via A/C and the CAM respectively, while it was $10^{7.8}$/ml. EID_{50} when inoculated via Y/S.

Table 3: Revealed that the main target organs of replication and reisolation is the whole embryo since the virus titer was $10^{9.1}$/ml. EID_{50} while the titer was $10^{6.5}$/ml, $10^{6.6}$/ml. EID_{50} in both A/C and CAM respectively.

Table 4: Decleared that the virus reached its peak of growth at 72 hours post inoculation when inoculated via A/C or CAM. As the titer was $10^{9.1}$/ml and $10^{8.5}$/ml respectively, while after 120 hours it was dropped to $10^{6.83}$/ml, $10^{6.17}$/ml via A/C and CAM respectively.

Table 5: Showed that there was no loss in virus titer when the harvested fluid was held for 1 hour at +10°C while the loss in titer was log 0.8, 0.8, 1.7 and 5.8 when held for 1 hour at 25°C, 37°C, 40°C and 56°C respectively.

Table 6: cleared the results of titration of the freez-dried vaccine samples when stored at different temperature degrees, room temperature, refrigerator and deep frrezer. It was found that there was loss in virus titer from $10^{8.3}$/ml to $10^{2.2}$/ml when kept at room temperature for 7 months from (April to October). When the temperature was ranged between 25°C - 35°C (April - June) the virus dropped to $10^{7.5}$/ml, and when the temperature reched 37°C, the virus dropped to 6.1 at July while there was a sharp dropped of the virus titer when the temperature was 40°C in August since it dropped to $10^{4.8}$/ml and there was a decline linear in dropping of the virus titer till it reached to $10^{2.2}$/ml in October after 7 months storage. The result of monthly titration of thevaccine samples kept at 4°C.

Table 7: Showed that after 9 months storage the reduction of virus titer was from $10^{8.3}$/ml to $10^{6.3}$/ml. There was no significance loss in the virus titer when it was kept at -20°C since after more than 1 year, there was no signicicance loss in the virus titer than the original one.

DISCUSSION

DHV infection among Egyptian duck farms is established as an involving disease which require well designed vaccination and controlling programs. FARID et al. (1972) and MOSTAGIR et al. (1972) isolated and identified the viral agent causing DHV in duckling in Egypt.

This study is the first step in trials to produce a local DHV vaccine. The results in tables (1 and 2) recorded the pathogenicity of DHV to ECE via different routes and the best route of inoculation. It was found that the A/C, CAM and Y/S gave 100%, 90% and 75% embryonic deaths respectively when inoculated with DHV vaccinal strain 10^{-6} dilution. Table (1) also showed that the number of dead embryos increased at 72 hours P.I. in all the 3 routes. TOTH (1969) found that the first embryo death was at 45th hour, the mortality increased at the 53 hours and by the 93th hours it was 100% when inoculated via A/S. HWANG (1965) recorded that the DHV kills embryos in dilution of 10^{5} to 10^{6} with a peak mortality at 48-60 hours P.I.

Fig. 2 & 3: Showd that the embryos that died 48-96 hours post-inoculation showed skin haemorrhage allover the body and oedema may be noted specially around the thigh and abdomen, while the embryos that died after 5-6 days P.I. showed typical lesions of DHV: skin haemorrhage may be present and oedema noted specially around the thigh, abdomen and head and neck, the yolk look greenish colouration and reduced in size, the liver showing witish yellow necrotic foci varying from pin head in size to larger area. Similar finding was found by LEVINE (1972).

Table (2) cleared that there was no significance difference in the titer of the vaccine when inoculated via CAM or A/C as the titer reached to $10^{8.6}$/ml - $10^{8.3}$/ml (EID_{50}) via CAM and A/C respectively, while it reached to $10^{7.8}$/ml. EID_{50} via Y/S route of inoculation. Experiment (3) was carried out for determination of the target organs for DHV multipliction. The whole embryos minus head and feet suspension. CAM suspension and AAF were examined individually. It was found that the embryo minus (head & feet) suspension was given the highest virus titer as it reached to 10^{9} (EID_{50}/ml) while in CAM suspension and AAF it was $10^{6.6}$-$10^{6.5}$ (EID_{50}/ml) respectively. This finding may be attributed to the presence of liver in the embryo as it considered the most suitable site for multiplication and the best organ for virus isolation LEVINE (1972). HWANG and DOUGHERTY (1964) found that the highest duck hepatitis virus concentration was found in the inoculated chicken embryos minus head and foot. Similar finding was recorded by MANSON et al. (1972) and WACHENDERFER (1966).

DUCK VIRUS HEPATITIS VACCINE

TOTH (1969) followed the growth of chicken embryosadapted virus (80 pasages 9 in three portions of the embryos found that titers were highest in embryo suspension ($10^{7.5}$ EID_{50}/0.1 ml) while in CAM suspension and AAF, it was $10^{5.79}$-$10^{3.62}$ EID_{50}/0.1 ml respectively.

Table 4: Showed that the most suitable time for harvest was at 72 hours P.I. which gave a titer of $10^{9.1}$ EID_{50}/ml and it was 10^{8}/ml at 48, 96 and 120 hours P.I. respectively.

TOTH (1969) concluded that a higher titer live vaccine could be harvested from the three component of the egg 53-69 hours P.I. and it may be possible to harvest at 77 hours P.I. Similar finding were reported by PAN (1981). MANSON et al. (1972) who noted that the higher titer reached its peak at 48 hours.

Table 5: Summarized the results of thermostability of DHV in the suspension of homogenized whole embryos minus head and feet, CAM and AAF after holding period of one hour at +10°C, 25°C, 37°C, 40°C and 56°C. The ID_{50} of DHV was droped to 5.8, 1.7, 0.8, 0.8, 0 log when held at 56°C, 40°C, 37°C, 25°C and 10°C for one hour respectively. Similar finding was found by HANSON et al. (1964) who found that the ID_{50} of DHV at 56°C decreased rapidly between 30 and 90 minutes, with 99% of the activity lost in 30 minutes and non detectable after 90 minutes.

ASPLIN (1961) reported that it would survive at 56°C for 30 minutes. HANSON et al. (1964) noticed that exposure of DHV to 37°C reduced active virus markedly after 48 hours, while the virus exposed to 25°C survived with little loss in activity.

The results in table (6) revealed that after 7 months storage of a freeze-dried vaccine samples kept invarious degrees at room temperature (from April to October) the average reduction of thevirus titer was 6.1 log_{10}. When the room temperature was 40°C the ID_{50} of DHV decresed rapidly and it was $10^{3.7}$ in August till reached to $10^{2.2}$ in October. While there was no loss in virus titer occurred when the room temperature was 25°C. HANSON et al. (1969) found that tissue suspension held at room temperature (23°C) and tested for viability over 150 days showed gradual linear decline in titer from 10^{8} to $10^{2.16}$/ml. Our results showed decreased in titer from $10^{8.3}$ to $10^{2.2}$/ml in 7 months. The difference in keeping quality of our results compared with the result obtained by HANSON et al. (1964) was due to the use of freeze-dried vaccine plus 10% skimmed milk as stabilizer.

Our results of monthly titrations of dried vaccine samples kept at 4°C showed that there was a reduction in virus titer from $10^{8.3}$ to $10^{6.3}$/ml after keeping for a period of 9 months (Table 7).

HANSON et al. (1964) found that suspension held at 4°C and tested over 52 weeks showed a titer decline from 10^{8} tp $10^{5.8}$/ml. ASPLIN (1961) found that the virus survived over 2 years at 4°C.

Our results showed that there was no loss in virus titer when stored at -20°C for more than 12 months. HANSON et al. (1964) found that the virus survived as long as 9 years at -20°C.

From the previous results recommended to produce a high titer live vaccine, it is reasonable to inoculate the seed material via CAM or A/C and to harvest the three parts of infected ECE (the whole embryo minus head and foot plus CAM and AAF) after 72 hours post inoculation on and it is recommended that all freeze-dried DHV vaccine should be stored in deep freezer as there was no significance loss in the virus titer after more than 12 months.

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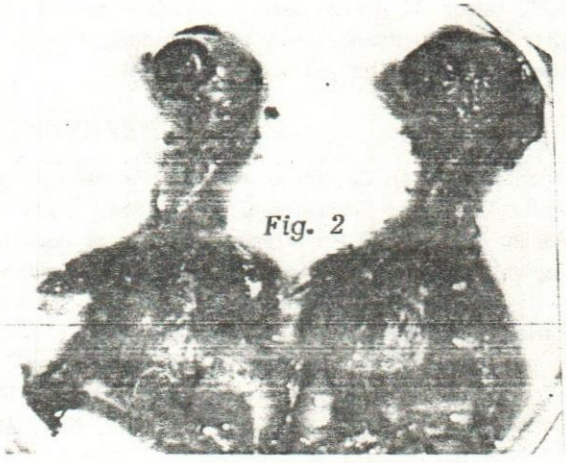

Fig. 1: Normal chicken embryo.

Fig. 2: Notice oedema around head and thigh.

Fig. 3: Notice congestion and greenish colouration of the liver.

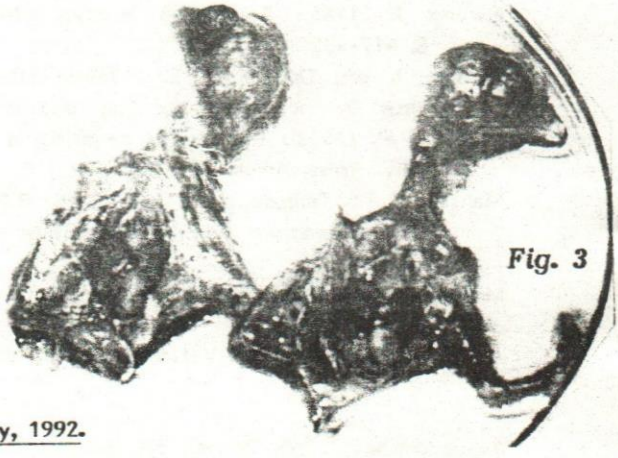

Table (1)

Determination of pathogenicity of DHV to ECE via different route of inoculation

Route of inoculation	Age of ECE	Pattern of embryonic deaths						% of pathogenicity
		24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.	
A/C	9 days	-	4	8	5	3	-	100%
CAM	10days	-	4	8	4	1	-	90%
Y/S	6 days	-	3	7	2	2	1	75%

Every route was inoculated into 20 ECE.

Table (2)

The effect of different routes of inoculation on DHV titer.

Route of inoculation	Virus titer in ECE EID_{50}/ml.
CAM	$10^{8.6}$
A/C	$10^{8.3}$
Y/S	$10^{7.8}$

Table (3)

Determination of the target organs for DHV multiplication

Type of organs	EID_{50} /ml. in egg
Whole embryo(minus head and foot)	$10^{9.0}$
CAM	$10^{6.6}$
AAF	$10^{6.5}$

Table (4)

Determination of the best time for harvesting of DHV vaccine via A/C and CAM

Hours post inoculation	Route of inoculation	
	allantoic cavity EID_{50}/ml.	chorio-allantoic membrane EID_{50}/mL
24	-	-
48	$10^{8.0}$	$10^{8.78}$
72	$10^{9.1}$	$10^{8.5}$
96	$10^{7.8}$	$10^{7.83}$
120	$10^{6.83}$	$10^{6.17}$

Table (5)

Determination of the thermostability of undiluted DHV fluid at various temperature degrees after a holding period of 1 hour

Temperature degrees	Titer of DHV before inactivation (EID_{50}/0.1ml.)	Titer of DHV after inactivation (EID_{50} /0.1 ml.)	Loss in virus titer (log_{10})
+10°C	$10^{8.3}$	$10^{8.3}$	0
25°C	"	$10^{7.5}$	0.8
37°C	"	$10^{7.5}$	0.8
40°C	"	10^{6}	1.7
56°C	"	$10^{2.5}$	5.8

Table (6)

Results of keeping quality of freez - dried DHV stored at room temperature as determined weekly

Temp. degree	Titer of DHV before storage	virus titer post storage weekly intervals																									
		April				May				June				July				August				Sept.				October			
	EID_{50}/ml.	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W	1 W	2 W	3 W	4 W
Room temp.	$10^{8.3}$	25℃	28	25	25	28	25	25	28	32	32	35	35	36	37	37	37	40		38	35		33	32		29		25	
		8.3	8.3	8.2	8.3	8.2	8.3	8.3	8.3	7.8	7.5	7.5	7.5	6.8	6.6	6.6	6.1	4.8	ND	3.7	3.5	ND	ND	3.2	ND	2.8	ND	2.2	0

W = week ND = Not done

Table (7)

Results of keeping quality of freez - dried DHV vaccine stored at +4°C and -20°C

Temp. degrees	titer of DHV before storage	Virus titer post storage monthly intervals								
		April	May	June	July	August	Sep.	Oct.	Nov.	Dec.
+4°C	$10^{8.3}$/ml.	8.3	8.3	8.3	8.0	8.0	7.5	6.6	6.8	6.3
-20°C		8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.1