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Publication/Creation

[Place of publication not identified] : [publisher not identified], [1926?]

Persistent URL

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Veterinary Biological Products—Their Standardisation and Application.*

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This paper was written for presentation to the North of England Division of the N.V.M.A., because, in choosing a subject on which to write, we have been guided by the facts that we could best give information on animal diseases which we have been investigating recently, and that all veterinary practitioners might not have full knowledge of the degree of accuracy with which veterinary biological products can be standardised. Our intention is to deal especially with biological products in use for the prevention, cure or diagnosis of blackleg in cattle and sheep, braxy in sheep, *B. bronchisepticus* infection in dogs, swine erysipelas, leptospiral canine jaundice, tetanus and tuberculosis. On a given batch many tests are usually made; we show only typical ones taken from our records.

BLACKLEG IN CATTLE AND SHEEP.

Because it has been shown that *Vibrion septique* is found alone or in association with *B. chauvæi* in some cases of clinical blackleg in cattle and sheep, products have been devised which, on subcutaneous injection, will confer immunity against infections of both organisms. This product consists of equal quantities of *B. chauvæi* filtrate which, in our experience, has proved atoxic for all animals even when injected intravenously in large amounts, and a toxin-antitoxin mixture of *Vibrion septique*, which is also atoxic, although *Vibrion septique* toxin alone is highly pathogenic. Many experiments have been done to show that this product does confer immunity (e.g., Allen and Bosworth, *Vet. Journal*, Vol. 80, No. 9) in which guinea-pigs and sheep have been used.

Tables I to IV illustrate results obtained by us in work on a recently made batch of this product. Table I shows that a potent *Vibrion septique* toxin had been produced of which 0.001 c.c., injected intravenously, was sufficient to kill a mouse. The titration of this toxin against *Vibrion septique* antitoxin was carried out as indicated in Table II, which shows that the toxin used was a specific one and that it was necessary to add 50 c.c. antitoxin per litre to neutralise the toxin. Table III indicates that this batch of product conferred immunity against *B. chauvæi* infection in guinea-pigs, and in Table IV are results of similar experiments in which *Vibrion septique* toxin was used to test the immunity.

It may be of interest to know that sheep injected with a similarly-made product have been shown to develop an immunity against *B. chauvæi* and *Vibrion septique* infections, which conferred full protection against virulent culture injected eighteen months later. It is fairly certain that this immunity lasts for many years and probably for life.

BRAXY IN SHEEP.

We have already shown that this *chauvæi-septique* prophylactic confers immunity against naturally occurring braxy (see *V. R.*, No. 28, Vol. 5). Since these results were published, we have had information on a small test carried out on sheep in Wales by Mr. N. Bisset, M.R.C.V.S., University College, Cardiff. One hundred young sheep were injected during the late autumn of 1924 with the preventive, and one hundred control sheep were kept under exactly similar conditions. The results at the end of the braxy season, 1924-25 were, that of the one hundred control sheep, eighteen had died of braxy, while of the one hundred inoculated ones, only one died.

TABLE I.

TITRATION OF VIBRION SEPTIQUE TOXIN.

Mice used—injections intravenously.

Toxin.	Result.
0.25 c.c. to 0.01 c.c.	All died.
0.005 c.c.	D.
0.001 c.c.	D.
0.0005 c.c.	L.

D = died. L = lived.

TABLE II.

TITRATION OF VIBRION SEPTIQUE ANTI-TOXIN.

Mice used—injections intravenously.

Toxin.	Serum.	Result.
0.01 c.c. ...	0.001 c.c. ...	L.
0.02 c.c. ...	0.001 c.c. ...	L.
0.03 c.c. ...	0.001 c.c. ...	D.
0.04 c.c. ...	0.001 c.c. ...	D.

Neutral point — 0.02 toxin = 0.001 c.c. serum.

Toxin and serum mixed and stood one hour before injecting mice intravenously.

L. = lived. D. = died.

TABLE III.

PROTECTIVE VALUE OF *B. CHAUVÆI* FILTRATE.

Four guinea-pigs injected with mixture containing 50 per cent. *B. chauvæi* filtrate.

Two doses given at interval of eleven days.

Test done of living culture five weeks later intramuscularly.

*Paper read before the North of England Division of the N.V.M.A. by Mr. T. Dalling, December 5th, 1925.

	Dose.				Result.
	1.0 c.c.	L.
	1.0 c.c.	L.
	0.5 c.c.	L.
	0.5 c.c.	L.
Control g.-ps. no filtrate— test only.	1.0 c.c.	D.
	0.5 c.c.	D.
L. = lived.	D. = died.				

TABLE IV.

PROTECTIVE VALUE OF VIBRION SEPTIQUE TOXIN-ANTI-TOXIN MIXTURE (NEUTRAL).

Four guinea-pigs each injected with 2 c.c. mixture containing 50 per cent. *Vibrio septique* toxin-antitoxin.

Two doses given at fourteen days' interval.

Test done of toxin intravenously four weeks later.

	Toxin.				Result.
	0.25 c.c.	D.
	0.25 c.c.	L.
	0.1 c.c.	L.
	0.1 c.c.	L.
Control g.-ps.	0.25 c.c.	D.
No T.A.M.	0.1 c.c.	D.
Test only	0.05 c.c.	D.
L. = lived.	D. = died.				

B. BRONCHISEPTICUS INFECTION IN DOGS.

We have already shown that, while the injection of guinea-pigs with killed cultures of *B. bronchisepticus* was followed by some immunity against subsequent lethal doses of the organism, the use of killed, followed by one dose of living, culture produced complete immunity more rapidly. The method adopted to produce immunity in dogs has, therefore, been the

injection of two doses of killed, followed by one dose of living vaccine. We wished to ascertain whether the same degree of immunity would be produced if the dose of living culture used had been prepared and allowed to stand some days before injection. A large experiment was undertaken to determine this point. Table V shows the details and results. The earliest immunity is found in cases where the living vaccine is freshly prepared, also, the use of living vaccine which has stood for seven days before use in amounts corresponding to those of freshly-prepared culture, does not confer such a high degree of immunity as is obtained by using a larger dose of killed culture as a final injection.

Table VI is produced to show the titration of an anti-bronchisepticus serum recently made.

TABLE VI.

B. BRONCHISEPTICUS SERUM.

Guinea-pigs used.		Injections intraperitoneally.		
Serum.		Culture.		Result.
2.0 c.c.	...	5 c.c.	...	L.
1.5 c.c.	...	"	...	L.
1.0 c.c.	...	"	...	L.
0.5 c.c.	...	"	...	L.
0.25 c.c.	...	"	...	L.
0.1 c.c.	...	"	...	L.
0.05 c.c.	...	"	...	L.
N.H.S.	2.0 c.c.	"	...	D. 1.
	0.1 c.c.	"	...	D. 1.
No serum	—	"	...	D. 1.

Serum and culture mixed and left at room temperature one hour before injecting.

L. = lived. D. 1 = died after 24 hours.

N.H.S. = Normal Horse Serum.

TABLE V.

B. BRONCHISEPTICUS.

G.P.	1st Inoc.	2nd Inoc.	3rd Inoc.	Test.	Result.
1	1.0 c.c. killed cult. subcut.	2.0 c.c. killed cult. subcut. 7 days later	0.5 c.c. living cult. subcut. 7 days later	4.0 c.c. living cult. I.P. 3 weeks later	L.
2					L.
3					L.
4					L.
5					D.
6					L.
7					D.
8	Control test only.				D.
9	2 doses as above		2 doses as 1, 2 and 3	As above 4 weeks later	L.
10					L.
11					L.
12					L.
13					L.
14					D.
15					D.
16	Control test only.				
17	2 doses as above		2 doses as 1, 2 and 3	As above 5 weeks later	L.
18					L.
19					L.
20					L.
21					L.
22					D.
23					D.
24	Control test only.				D.

L = Lived.

D = Died.

SWINE ERYSIPELAS.

Standardisation of Serum.—Two methods can be employed in the standardisation of anti-swine erysipelas serum:—

(a) Mouse method, (b) Pigeon method.

Mouse method.—Cultures of *B. erysipelatis suis* are grown in broth for twenty-four hours, when more broth is added to form a dilution of culture of 1 in 30. Varying doses of the serum to be tested, from 0.03 c.c. to 0.005 c.c., are injected subcutaneously into mice, and one hour later 0.3 c.c. of the diluted culture is injected intraperitoneally. Two mice are used for each dose of serum and deaths are recorded for seven days. Table VII illustrates the testing of a sample of serum by this method and a comparison with a standard serum, the results of which indicate that the test serum is of much higher value than the standard.

TABLE VII.

SWINE ERYSIPELAS SERUM.

Standardisation by Mouse Method.

Test Serum.	Culture.	Result.
0.005 c.c. ...	0.3 of 1/30 dil. ...	L. D. 7.
0.008 " ...	" ...	L. D. 7.
0.01 " ...	" ...	L. L.
0.015 " ...	" ...	L. L.
0.02 " ...	" ...	L. L.
0.03 " ...	" ...	L. L.
<i>Standard Serum.</i>		
0.005 c.c. ...	0.3 of 1/30 dil. ...	D. 4. D. 4.
0.008 " ...	" ...	D. 3. D. 4.
0.01 " ...	" ...	D. 4. L.
0.015 " ...	" ...	D. 4. L.
0.02 " ...	" ...	L. L.
0.03 " ...	" ...	L. L.
<i>Control Mice.</i>		
No serum ...	0.3 of 1/30 dil. ...	D. 3. D. 4.
Serum injected subcutaneously one hour before culture injected intraperitoneally.		
D. 3., etc. = died on 3rd day, etc. L. = lived.		

Pigeon method.—The M.L.D. for pigeons of a culture of the organism is established, injections being made intramuscularly. Serum in amounts of 0.1 c.c., 0.2 c.c. and 0.3 c.c. is then mixed with 500 M.L.D.'s of the culture and the whole injected intramuscularly. A sample of standard serum is tested in parallel. Table VIII indicates that 'X' serum is at least equal to standard, while 'Y' is below standard in value.

TABLE VIII.

SWINE ERYSIPELAS SERUM.

Standardisation by Pigeon Method.

Serum.	Dose of Serum.	Culture.	Result.
(X)	0.3 c.c.	500 M.L.D.	L.
	0.2 "	"	L.
	0.1 "	"	D. 4.
(Y)	0.3 "	"	L.
	0.2 "	"	D. 3.
	0.1 "	"	D. 3.

Serum.	Dose of Serum.	Culture.	Result.
Standard	0.3 c.c.	500 M.L.D.	L.
	0.2 "	"	L.
	0.1 "	"	D. 4.
No Serum		"	D. 3.
		100 "	D. 4.
		10 "	D. 4.

Serum and culture mixed and injected intramuscularly.
L = lived. D. 3. = pigeon died 3rd day.

Active Immunity.—Two main methods for the production of active immunity in pigs against swine erysipelas, are in practice dependent on whether or not infection is present in the premises. The 'living culture' method is adopted on clean premises, while, where the disease already exists, the serum-virus method is practised. In the 'living culture' method, two doses of a living culture of the organism are injected at an interval of 7-10 days, no serum being used. The serum-virus method involves the injection of a living culture of *B. erysipelatis suis* and at the same time a dose of serum, followed in 7-10 days by an injection of living culture.

In our attempts to standardise vaccines for use in pigs, we have made use of pigeons for our preliminary work. Satisfactory pigeon methods will be tested in pigs. Table IX is the result of two such pigeon experiments in which (a) living cultures alone have been used, the result being that four out of seven pigeons were immune when tested four weeks after injecting, (b) the serum-virus method was adopted, after which four out of six pigeons treated resisted infection when tested.

TABLE IX.

SWINE ERYSIPELAS.

Active Immunity.

Pigeons used.

A. <i>Living Culture.</i>				
Pigeon.	1st. Inoc.	2nd. Inoc.	Test.	Result.
1	0.0005 c.c. living culture subcut.	0.0005 c.c. living cult. subcut. 7 days later	0.01 c.c. living cult. intramuscu- larly 4 weeks later.	L.
2				D.
3				D.
4				L.
5				L.
6				L.
7				L.
8	Control—No vaccine.			D.

B. <i>Serum and Virus followed by Virus.</i>				
Pigeon.	1st. Inoc.	2nd. Inoc.	Test.	Result.
1	0.001 c.c. living cult. + 0.01 c.c. serum subcut.	0.005 c.c. living cult. 7 days later.	0.01 c.c. living cult. 4 weeks later.	D.
2				D.
3				L.
4				L.
5				L.
6				L.
7	Control—No vaccine.			D.

L. = lived. D. = died.

LETOSPIRAL CANINE JAUNDICE.

In the *Veterinary Journal* (Vol. 81, No. 1) was published an article by Okell, Dalling and Pugh, indicating that malignant or infectious jaundice in dogs can be produced by *Leptospira icterohæmorrhagicæ* and that an efficient antiserum had been produced. Such a serum is titrated in guinea-pigs. To show that this serum will protect dogs, even after infection has taken place, an experiment, shown in Table X, was undertaken. Of the dogs treated, dogs 1-6, which had been injected with the serum up to the fourth day after infection, remained healthy, showing no symptoms of jaundice. Serum was injected into dogs 7-10, ninety-six hours after infection, at which stage icterus was marked. All died from well marked malignant jaundice, as well as dog 11, which had received no serum. *Post-mortem* examinations confirmed the diagnosis of the disease. It is thus evident that antileptospira serum can protect dogs even after infection has taken place; its greatest use, however, is in preventing the spread of the disease to in-contact dogs in infected kennels.

TABLE X.
ANTILEPTOSPIRA SERUM.
Dog Experiment.

Dogs I—XI inoculated with virulent dog strain of leptospira intraperitoneally.

Dogs I and II	10 c.c. serum 24 hours later	L. L.
„ III and IV	„ „ 48 „ „	L. L.
„ V and VI	„ „ 72 „ „	L. L.
„ VII, VIII, IX, X	„ „ 96 „ „	D. D. D. D.
„ XI	No serum	D.
L. = lived. D. = died and showed typical P.M.		

ACTIVE IMMUNITY.

Japanese and other workers have stated that Weil's disease (leptospiiral jaundice in the human subject)

can be prevented by the use of vaccines composed of killed cultures of leptospiræ. We have attempted to immunise dogs by vaccination, the vaccines consisting of the ground-up livers of guinea-pigs which were infected with leptospiræ, treated to destroy the organism. Some preliminary laboratory experiments were carried out on puppies, the results of which led to a more extensive laboratory test. The results are shown in Table XI and indicate that complete protection was obtained after three injections of vaccine. The immunity test was severe, *viz.*, 1 c.c. of ground-up guinea-pig liver rich in virulent leptospiræ was injected. This was probably many times the dose necessary to infect a dog artificially and much more than any dog receives under natural conditions.

Field trials have been in progress for some time. The results of two trials are shown in Table XII, in both of which the control (uninoculated) and the inoculated groups of dogs were kept under exactly similar conditions. No special animals were chosen for either group. It is interesting to note that the inoculated dogs which died of leptospiral jaundice were of a group which had received one inoculation only.

TABLE XII.
LEPTOSPIRA VACCINE.
Field Results.

Group A.

Control Dogs.	Died.	Per cent.	Inoculated.	Died.	Per cent.
34	8	23.5	94	0	0

All dogs had two inoculations at seven days interval

Group B.

Control Dogs.	Died.	Per cent.	Inoculated.	Died.	Per cent.
17	8	47	35	2	5.7

Of the thirty-five dogs, twenty-eight had only one inoculation (two of these died). Seven had two inoculations (none died).

TABLE XI.
LABORATORY EXPERIMENT ON DOGS.

Group A.						
Dog.	1st Inoc.	Interval.	2nd Inoc.	Interval.	Test.	Result.
19	1 c.c. phenolised g.-p. liver	7 days	As 1st	5 weeks	1 c.c. virulent g.-p. liver	L.
21						D.
36						Control—No vaccine
Group B.						
Dog.	1st Inoc.	2nd Inoc.	3rd Inoc.	Test.		Result.
37	1 c.c. phenolised g.-p. liver	As 1st 7 days later	As 1st 5 weeks later	1 c.c. virulent g.-p. liver 3 weeks later		L.
38						L.
39						L.
42						L.
Controls.						
61	No vaccine—Test only.					D.
62						D.
63						D.
64						D.
65						D.
66						D.
L = Lived. D = Died. P.M. Typical.						

L = Lived. D = Died. P.M. Typical.

TETANUS.

Standardisation of Antitoxin.—Tetanus antitoxin is used largely as a prophylactic agent against tetanus in horses; varying success is claimed by veterinary practitioners for its use as a curative in affected animals. Because of the amount of research work that has been done on tetanus toxin and antitoxin, standardisation can be done with great accuracy. For such work a standard toxin is necessary, which, after testing, is dried to a powder and kept for future use. In the experiment shown in Table XIII, 0.00066 gram was found to be the amount of dry toxin neutralised by 0.1 unit of a standard antitoxin (the unit of standard antitoxin being ten times the amount required to delay deaths in a guinea-pig of 350 grams weight for ninety-six hours after the injection of 100 M.L.D. toxin). It will be seen from the table that 0.008 c.c. of the test serum is sufficient to neutralise the test dose of toxin, i.e., 0.0008 c.c. test serum is equivalent to 0.1 of a unit of standard antitoxin. In other words, the serum contains 1250 units per 10 c.c.

TABLE XIII.

TITRATION OF TETANUS ANTITOXIN.

Guinea-pigs of 350 gram weight used.

Toxin.	...	Serum.	...	Result.
0.00066 gm.	...	0.0006 c.c.	...	D. 48.
"	...	0.0007 c.c.	...	D. 90.
"	...	0.0008 c.c.	...	S.
"	...	0.001 c.c.	...	S.

Three tests done on each serum. 0.0008 c.c. serum = 0.1 unit, therefore serum = 125 units per c.c., i.e., 10 c.c. = 1250 units.

D. 90 = died of tetanus in ninety hours. S. = survived.

It has been shown by long experimentation that tetanus antitoxin deteriorates only at the rate of 5 per cent. per year if kept at ordinary temperature in the dark, and hence one can readily calculate the value of antitoxin after a number of years, being given its original value in units.

ACTIVE IMMUNITY.

Active immunity against tetanus has not been practised to any extent, yet a high degree can be secured. Buxton and Glenny (see *Lancet*, 1921, ii, 1109) have shown that horses containing no trace of normal tetanus antitoxin, when injected with definitely over-neutralised toxin-antitoxin mixture in three doses at three days intervals, have, one month later, resisted a dose of toxin of which 1/2000 part would kill a guinea-pig. They also showed that four horses immunised as above were able, two months later, to tolerate 2 c.c. of actively growing *B. tetani* culture mixed up with sterile garden soil and injected intramuscularly. Further, in immunising horses in the production of tetanus antitoxin, one dose of over-neutralised toxin-antitoxin mixture is given. Some weeks later a horse so injected will tolerate a large amount of crude tetanus toxin sufficient to kill any untreated horse.

TUBERCULOSIS.

Standardisation of Tuberculin.—Several methods are used for the standardisation of tuberculin. At the

Wellcome Physiological Research Laboratories a double guinea-pig test is adopted, (a) intradermic test, (b) subcutaneous test (*vide* Eagleton, *Lancet*, 1921, i, 429).

A number of guinea-pigs are injected with virulent living T.B. culture and are examined at intervals for evidence of infection. Table XIV summarises the methods. When infection is believed to have advanced sufficiently, a *preliminary intradermic test* is carried out, i.e., a standard tuberculin is diluted in saline to 1-500; 1-1000; 1-2000, and 0.2 c.c. of each is injected intradermally into areas from which the hair has been removed. If definite reactions are found twenty-four hours later, the *main intradermic test* is carried out in which lower dilutions of the standard tuberculin are made with a corresponding series of dilutions of the test tuberculin. All dilutions are injected on the same animals and thus true comparative readings can be made between the standard and test tuberculin. The advantage of the intradermic method of standardisation is that the same animal shows the reactions of the test and the standard tuberculin, and the degree of difference between standard and test tuberculin can be ascertained.

The subcutaneous test can now be carried out. A *preliminary test* is done, using standard tuberculin. When guinea-pigs are sufficiently sensitive, 0.1 c.c. should kill 50 per cent. of those injected within twenty-four hours. The *main subcutaneous test* is now undertaken, when different animals are injected with corresponding doses of standard and test tuberculin. If the tuberculin are equal, the death rate amongst the injected guinea-pigs will be equal. The test is not such a delicate one as the intradermic.

TABLE XIV.

STANDARDISATION OF TUBERCULIN.

Fifty guinea-pigs injected with living virulent T.B.

A. After ten days. *Preliminary Intradermic Test.*

0.2 c.c. standard tuberculin diluted.

1/500, 1/1000, 1/2000.

B. *Main Intradermic Test.*

1/1000, 1/2000, 1/4000, 1/8000.

Standard tuberculin...	+	+	+	+	±	±	±
Sample tuberculin ...	+	+	+	+	±	±	±

C. *Preliminary Subcutaneous Test.*

Standard tuberculin injected.

0.25	+
0.1	+
0.1	S.

D. *Main Subcutaneous Test.*

	Tuberculin.	Dose.	Result.
Standard	...	0.1	...
		0.1	S.
Sample	...	0.1	...
		0.1	S.

† = died within twenty-four hours. S. = survived

+ + ± ± ± ± = Degrees of reaction.

DISCUSSION.

The PRESIDENT, in opening the discussion, said they were grateful to Mr. Dalling for the time and trouble he had expended in the preparation of his paper and for the clear manner in which he had explained the methods of standardisation. The subject matter was of such interest that he would call upon each member present to take part in the discussion.

Mr. C. ELPHICK felt that they had had an intellectual feast. They could now clearly see how the laboratories were helping them with their various troubles, and they were better able to appreciate the care that was expended to ensure that their products were of a standard potency.

He wished to ask Mr. Dalling a few questions.

(1) Was leptospiral jaundice the same disease as that known as "The Yellows," and was that a form of distemper?

(2) How could they diagnose leptospiral jaundice?

(3) Why did the intradermic tuberculin not react in one case; also how was the eye reaction accounted for?

Mr. DAVIDSON added his note of appreciation to the lecture. He had found the intradermic tuberculin test reliable.

In cases of tetanus, what number of units of anti-serum did Mr. Dalling suggest as appropriate? He had used 3,000 units, only to find that the animal died.

Mr. JACKSON YOUNG expressed great pleasure at hearing Mr. Dalling and said each one should try for himself the products which had been mentioned. He was particularly interested to hear that blackleg vaccine gave immunity for at least twelve months.

Referring to braxy, he said that one preparation contained garlic; this, of course, should not be used.

He would like to know the length of the time considered appropriate for pigs injected with swine erysipelas serum to remain in contact with infected animals. His own view was that, in the case of swine erysipelas, hygiene was of greater importance than serum.

Mr. Young was further interested to know whether there was any advantage to be gained in preparing tuberculin from the strain of organism in this country.

Mr. HILL asked the dosage which Mr. Dalling would advise when using tetanus anti-serum. In the treat-

ment of tetanus cases he had obtained better results without the use of serum. Like Mr. Elphick, he was anxious to learn how to diagnose leptospiral jaundice.

Speaking of blackleg, Mr. Hill had had excellent results from the vaccine. He had never had a death following its use.

Mr. FORBES had had better results in tetanus cases when large doses of anti-serum had been used. He had always found blackquarter vaccine to give excellent results.

Mr. STARKEY put forward a problem. He had never had sucking puppies affected with distemper, or sucking pigs with swine erysipelas. He bred puppies in his distemper kennels and never had trouble. He would like Mr. Dalling to give the reason for this experience.

Mr. TAYLOR hoped Mr. Dalling, having once visited the North of England Division, would return again to give them more information on laboratory methods.

In tetanus cases he had given up the use of anti-serum as a curative measure.

Mr. THOMPSON said he had derived no benefit from the use of tetanus anti-serum as a curative agent.

Mr. DALLING, in his reply, said that leptospiral jaundice was characterised by a gastro-intestinal inflammation. There was no biological test for the disease.

The reason for the ophthalmic reaction to tuberculin was to be found in the extreme sensitivity of the eye.

Tetanus anti-serum was of little value in the severe case, where there was much damage to nerve tissue. It would help to counteract toxin in the circulation.


In reply to Mr. Jackson Young, he said he had isolated the *Vibrio septique* from cases of blackleg. He did not think that the strain of tubercle bacillus used in the making of tuberculin would have much effect upon its diagnostic value.

Mr. Starkey's problem depended on the fact that the young animal received immune bodies from the mother. These were elaborated by the brood bitch and passed on to the young in the milk.

The PRESIDENT proposed a hearty vote of thanks to Mr. Dalling for his address, and to Messrs. Broadhead and Sargeant for their kindness in working the lantern.

The proposal was seconded by Mr. DAVIDSON, and carried with acclamation.





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