A COMPARATIVE STUDY OF EQUINE PREGNANCY TESTS USING THE GALLI-MAININI AND THE ASCHHEIM-ZONDEK REACTIONS

By

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During recent years considerable progress has been made in the biological diagnosis of pregnancy in mares.

It is well-known that during the first 40 days of gestation there are no gonadotrophic hormones in the blood of pregnant mares: From about the 45th to about the hundredth day after serving, however, gonadotrophins are easily detectable in the bloodserum by biological tests, for example the Aschheim-Zondek reaction (A.-Z. reaction, 1928) and the Galli-Mainini test (G.-M. test, 1947). The latter refers to the release of sperm cells from the testis tubules of Amphibians by gonadotrophins and other substances, a process which has been termed spermiation by van Oordt & Klomp (1946).

As early as 1948 Del Pero applied the G.-M. test to mares, but he considered it useless, as only 3 positive results were obtained in 11 pregnancy cases. On the other hand Juhasz & Dozsa (1950) obtained a 100 % agreement with the A.-Z. reaction in 100 tests.

Between these extremes practically all intermediate evaluations exist. In one respect, however, the investigators agree: i.e. a positive G.-M. test indicates a 100 % certainty of pregnancy!

In view of the different results obtained with the G.-M. pregnancy test in mares, it was desirable to reinvestigate its usefulness and to compare its reliability with the A.-Z. reaction, particularly as the G.-M. test offers many

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advantages both in simplicity of procedure and in rapidity over the more laborious A.-Z. reaction.

M A T E R I A L  A N D  M E T H O D S

For the G.-M. test male green frogs (Rana esculenta) were used, weighing 30-70 gm., and possessing well-developed vocal sacs, the size of which is as a rule correlated with the size of the testes. The frogs were usually used several times, but in the tests, the question of whether the frogs were injected for the first time or had been used previously was taken into consideration. 217 blood samples of mares were investigated with the G.-M. test; 146 of these were apparently from pregnant mares; 62 were from non-pregnant mares and 9 samples were not suitable for investigation. 17 of the blood samples from pregnant mares originated from one experimental mare, which was tested from the 34th-135th day after coitus, and belonged to the Obstetrical Clinic of the Veterinary Faculty, University of Utrecht.

For the test bloodserum was used, as it was found that citrated plasma was toxic for the frogs. In cases in which chorionic gonadotrophin or serum gonadotrophin (resp. pregnyl and gestyl, Organon N. V.) were used the hormones were dissolved in 0.9 % saline.

For the G.-M. tests, the frogs were injected through the thigh-lymphsac into the dorsal lymphsac with 3 ml. of serum. Four hours later a urine sample was taken from the cloaca and investigated microscopically. If sperms were present the reaction was considered positive.

In investigations on the sensitivity of the frogs to different factors the »50 % dose of pregnyl«, i.e. the dose of pregnyl to which 50 % of the injected frogs reacted positively, was determined.

For the A.-Z. reactions the method of the Obstetrical Clinic of the Veterinary Faculty was used: for each test a batch of 5 immature female mice (body weight 8-10 gm.) was used, each of them receiving 6 subcutaneous injections of serum in the course of 3 days (on the first day 1, on the second, 3 and on the third day, 2 injections). One mouse received 1 ml., three mice 0.5 ml. and one mouse 0.2 ml. of serum per injection, i.e. a total of respectively 6, 3, 3, 3 and 1.2 ml. On the 6th day the mice were sacrificed.

Altogether 169 bloodserum-samples were examined. The reaction was considered positive if blood spots or corpora lutea were found, even if only one mouse reacted; in addition, two characteristic signs, namely enlarged ovaries and uterine horns, were always present. If only enlarged ovaries and uterine horns were found, this was taken as a positive indication of pregnancy. If one of these 4 characteristics did not develop, the reaction was considered negative. As it was found that bloodserum treated with ether was not as toxic for mice as bloodserum, the mice were injected with serum, previously shaken with ether.

Blood samples were received in 1952 from the second half of April to the first half of December, with a maximum during July and August.

R E S U L T S

1. The Galli-Mainini test

The blood samples from the 62 mares, which turned out to be non-pregnant, gave a negative G.-M. reaction in all cases.

The experimental mare, from which 17 blood samples were collected, gave
only positive results between the 44th and 111th day after the last service; between the 49th and 89th day maximum reactions were obtained. The results are shown in Fig. 1.

The other 129 blood samples collected between the 29th and 190th day after serving were from 124 pregnant mares; the blood of three mares was investigated twice, and that of one mare, three times.

In Fig. 2 the percentage of positively reacting frogs is plotted against the number of days after serving. The different dots show almost the same distribution as those of Fig. 1: the first reactions were obtained at about the 43rd day after the last coitus; between the 43rd and the 80th day after the last service the best results were obtained, 90.1% of the reactions being positive. From the 80th to the 120th day, the number of positive reactions decreased gradually and after the 120th day no positive reactions were observed.

The dots in Fig. 2 show a wide scatter, indicating a rather low sensitivity of the test animals. This variability in reaction may be caused by:

1. a variation in the response of the test animals,
2. a variation in the concentration of gonadotrophins in the bloodserum.

1. *Variation in sensitivity*. With regard to the sensitivity of the frogs, we have found that

a. There is an individual variation in their response to gonadotrophins. The threshold values in the sensitivity of the frogs for serum gonadotrophin vary from 50 to 10 l. U. and for chorionic gonadotrophin from 10 to 1.5 l. U.

b. The season at which the G.-M. test is carried out plays an important rôle. A distinct fluctuation in sensitivity of the frogs during the course of the year was clearly established (Fig. 3). These observations suggest that from
G.-M. tests. Percentages of positively reacting frogs, treated with bloodserum-samples of 124 pregnant mares. The solid line is the same as that of Fig. 1.

January onwards the sensitivity increases gradually until May and June, the months during which reproduction normally takes place. In July, however, the sensitivity decreases suddenly, and in August and September it is about half that of May and of June. In the last 3 months of the year the sensitivity increases again.

c. Frogs which are used for the first time are distinctly more sensitive than frogs which have already been used previously for the G.-M. test. The first are therefore preferable.

Seasonal variation in sensitivity of frogs to pregnyl. In May and June sensitivity is about twice as high as in August and September. (For 50% dose in I. U. pregnyl, cf. text p. 387).
2. *Variation in gonadotrophin concentration.* With regard to the variation in concentration of gonadotrophin in the bloodserum we would in the first place like to draw attention to the fact that the gonadotrophic hormone of the pregnant mares' bloodserum consists of about 20% of luteinizing hormone (LH) and of about 80% of follicle stimulating hormone (FSH). According to Crézé (1949) and other investigators spermiation is only evoked by LH.

As it follows from our observations (cf. Fig. 2) that the G.-M. test is only positive from the 43th to the 120th day after serving, it is accepted that only during this period is there a quantity of LH present, large enough to evoke spermiation.

Moreover, mares' bloodserum contains a principle which impedes spermiation. This is indicated by the fact that G.-M. tests, carried out with urine of pregnant human females give much quicker results than those carried out with bloodserum of pregnant mares. Human pregnancy urine usually evokes a positive reaction within an hour; after injection of bloodserum, however, spermiation is not obtained earlier than after 3 or 4 hrs. Since spermiation is evoked within an hour, after chorionic gonadotrophin injection (pregnyl, Organon) as well as after injection of serum gonadotrophin (gestyl, Organon), dissolved in physiological saline solution (0.9% NaCl), the difference in reaction time mentioned above, cannot be attributed to different properties of the gonadotrophic hormone, present either in human pregnancy urine or in pregnant mares' bloodserum. On the contrary, the cause of the different reaction times must be ascribed to the bloodserum *per se*, as the administration of pregnyl, dissolved in 0.9% NaCl evokes spermiation after 40–50 minutes, whereas pregnyl, dissolved in the serum of a non-pregnant mare, does not evoke spermiation before 120 à 200 minutes after injection. This is in agreement with an observation of Salvatierra & Torres (1952 a), who found that the dose of gonadotrophic hormone dissolved in serum of a non-pregnant woman and causing an identical reaction in male green frogs was 3 times higher than the dose of gonadotrophin dissolved in physiological saline solution. Presumably the limiting factor is due to the serum proteins present.

Finally we have investigated the possible effect of parity, that is the number of times that a mare has been pregnant, on the results of the G.-M. test. We divided all pregnant horses, Oldenburger as well as Belgian ones, into these groups:

1. Mares, which were pregnant for the first time (parity 1).
2. Mares, which were pregnant for the 2nd–5th time (parity 2–5).
3. Mares, which had been pregnant for more than 5 times (parity 6 or more).

The results, expressed as the mean percentage of positively reacting frogs, are summarized in Table 1.

According to Wilcoxon's test the P-values of the differences between groups 1 and 2, 2 and 3, and 1 and 3 were, for the Oldenburger and Belgian horses
Table 1.
Relation between parity and results of G.-M. tests in pregnant horses, 43-100 days after serving.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parity</th>
<th>Mean percentages of positively reacting frogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oldenburger horses</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>67.7</td>
</tr>
<tr>
<td>2</td>
<td>2-5</td>
<td>46.6</td>
</tr>
<tr>
<td>3</td>
<td>6 or more</td>
<td>11.4</td>
</tr>
</tbody>
</table>

together, respectively 0.01-0.04, 0.001-0.066 and <0.001. Hence it may be concluded that young mares, which are pregnant for the first time give much better results than older mares which have been pregnant more than once. Whether this phenomenon must be ascribed to the parity per se, or to the age of the mares, was not established.

II. The Aschheim-Zondek reaction
As far as the quantity of bloodserum allowed, parallel investigations were carried out, using the A.-Z. reaction.

To evaluate the presence of LH or FSH in the bloodserum the following signs in the test animals were used: the appearance of blood spots and corpora lutea (under the influence of LH) and the increase in size of the ovaries and of the uterine horns (under the influence of respectively FSH and oestrogen). Altogether 169 bloodserum-samples were tested, of which 112 were from 107 pregnant mares. The results are shown in Fig. 4.

The figures on the ordinate indicate the valuation estimations of the signs evoked by the bloodserum-samples. For each mouse showing blood spots and (or) corpora lutea (and if so also showing enlarged ovaries and uterine horns) the value 2 was awarded. Each mouse with only enlarged ovaries and uterine horns got only 1 point. The figures on the ordinate always relate to the total values awarded to the 5 mice, used for each A.-Z. reaction. Hence 5 is the maximum value possible, awarded to 5 mice which only show enlarged ovaries and uterine horns (FSH-signs) and 10 the value awarded to 5 mice, which all show blood spots and corpora lutea.

Moreover, Table 2 shows the percentages of positive and negative G.-M. tests and A.-Z. reactions in pregnant and non-pregnant mares.

From Fig. 2 and Table 2 it is seen that in pregnant mares the G.-M. test
yields 90% positive results between the 43rd–80th days after coitus, that between the 81st–120th days after serving this test is positive in about 50% and that after 120 days after serving no positive reactions were obtained.

In some cases the A.-Z. reaction, however, gives positive FSH-results before the 43rd day, when the effects of LH are not yet present. After the 43rd day negative results with the A.-Z. reaction are extremely rare, whereas false negative results are then fairly common with the G.-M. test.

The most important result, however, is that the A.-Z. reaction can be performed during a much longer period than the G.-M. test.

Using blood samples with different concentrations the FSH-factor in blood-

<table>
<thead>
<tr>
<th>Pregnancy-period (days after serving)</th>
<th>G.-M. test</th>
<th>A.-Z. reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% pos.</td>
<td>% neg.</td>
</tr>
<tr>
<td>29–42</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>43–80</td>
<td>90.1</td>
<td>9.9</td>
</tr>
<tr>
<td>81–120</td>
<td>51.2</td>
<td>48.8</td>
</tr>
<tr>
<td>121–190</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>non-pregnant</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.
Comparison of results of G.-M. tests and A.-Z. reactions in pregnant mares during different pregnancy-periods and in non-pregnant mares.
serum was first identified with the A.-Z. reaction (increase in size of ovaries and uterine horns); with higher concentrations LH-effects (blood spots and corpora lutea) were also obtained using this reaction. In the last case positive results were also achieved with the G.-M. test (78.2 %), but in 21.8 % no spermiation took place with this method.

We can thus say that the A.-Z. reaction is more sensitive to LH than the G.-M. test; it is also important that with the A.-Z. method, FSH can also be demonstrated. It must be emphasized, however, that with the bloodserum of non-pregnant mares FSH-effects (size increase of ovaries and uterine horns) are sometimes obtained (in 5.3 %).

Finally, we have found that increasing doses of bloodserum become more and more toxic to the mice: in 96 cases in which bloodsera of pregnant mares were injected into mice in doses of 1.2, 3 and 6 ml. the mortality was respectively 6.2, 16.3 and 41.7 %. With increasing doses the percentages of mice, reacting to LH did not increase much; the figures for doses 1.2, 3 and 6 ml. being 48.9 %, 56.4 % and 60 % respectively. Hence we consider a dose of about 3 ml. of bloodserum as representing the optimal dose for the A.-Z. reaction.

**DISCUSSION**

The G.-M. test is widely used in human pregnancy diagnosis for its many advantages: the test animals are easily obtainable and cheap, the method is very simple and the results, available within an hour, are highly reliable. Moreover, the test can be used from 2 weeks after the non-appearance of the expected menstruation until the end of the pregnancy period. In the mare, however, the G.-M. test offers several difficulties, which can only partly be overcome.

In the first place, rather large quantities of bloodserum are necessary. Moreover, no gonadotrophic hormone can be demonstrated in the serum before the 43rd day after serving and in the third place its concentration diminishes so much after the 80th day that the test becomes unreliable: between the 43rd and 80th days after serving negative reactions are by no means rare.

It appears therefore that the concentration of the hormone does not always exceed the threshold dose for sensitivity of the frogs' reaction. Also the percentage of LH in pregnant mares' serum is much smaller than in human pregnancy urine: in the mare about 20 % of the gonadotrophic hormone is LH, 80 % being FSH, whereas the gonadotrophic hormone in human pregnancy urine is almost all LH.

Moreover, mares' serum contains a principle which impedes spermiation (cf. also Salvatierra & Torres, 1952 a). Hence the reaction time is considerable slowed down.

In addition there is an enormous variability in the percentage of positively
reacting frogs. This is not only due to several factors which influence the reaction-capacity of the frogs (as e.g. whether the frogs are used for the first time or not, and the differences in sensitivity of the frogs in the course of the year), but also to changing gonadotrophin concentrations in the blood-serum.

It is interesting that in Southern Europe, e.g. in Spain (Salvatierra & Torres, 1952 b), the period with the lowest reaction-capacity of Rana esculenta occurs earlier in the year, i.e. during May, whereas the most sensitive period is during February and March. Lajos, Pali & Kummerländer (1951) did not find seasonal fluctuations in reaction-capacity in Hungary in this frog and therefore recommend Rana esculenta as experimental animal for quantitative gonadotrophin estimations. In North America Rana pipiens shows still larger seasonal fluctuations in sensitivity (Holyoke & Hoag, 1951; Biesinger & Miller, 1952).

Finally the parity or the age of the mare is of importance: Bloodserum of young mares, which had not been pregnant previously, gave much better results than the bloodserum of mares which had been pregnant one or more times previously.

In the literature some statements are made, according to which the breed of horse is of importance with regard to the concentration of gonadotrophic hormone (cf. Bielanski, Ewy & Pigoniowa, 1952, and Cole, 1948). The small number of data available to us, did not reveal any similar results.

All these factors are not in favour of applying the G.-M. test in equine pregnancy. Only between the 43rd and 80th day of gestation does this test give results with a certainty of about 90 %. If, however, the G.-M. test gives a positive result, then the pregnancy diagnosis is absolutely certain (100 %!).

The A.-Z. reaction is much more laborious, time consuming and expensive than the G.-M. test. If only the LH-effects (blood spots and corpora lutea) are taken into consideration, the A.-Z. reaction gives 92.6 % of positive results between the 43rd and 80th day after serving. On the other hand, blood from non-pregnant mares gives a negative LH-reaction of 100 %.

If the FSH- (enlarged ovaries and uterine horns) as well as the LH-signs are taken into consideration and considered as positive indications of pregnancy, then the A.-Z. reaction gives a 100 % positive reaction between the 43rd and 80th day after coitus. The FSH-effects, however, also appeared to develop in some cases after the injection of the bloodserum of non-pregnant mares (5.3 %!).

Taking all this into consideration, we recommend the following procedures:

1. The G.-M. test with mares' bloodserum should be carried out between the 43rd and 80th day after serving.
2. If the reaction is positive, then further investigations are superfluous. If it is negative, however, then the probability that the mare is not pregnant is 90 %. 

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3. If in this case more certainty is required, an A.-Z. reaction must be started on the same day and with the same material.

4. If using the A.-Z. reaction the LH-signs (blood spots and corpora lutea) as well as the FSH-signs (increase in size of ovaries and uterine horns) develop, then pregnancy can be demonstrated between the 43rd and 80th day after serving with a certainty of 100%, and between the 43rd and 190th day with a certainty of 97%. Bloodserum of non-pregnant mares, however, gives a negative result in 95% of the cases.

Our results are in agreement with those of Bentz (1951) who, like us, compared the G.-M. test with the A.-Z. reaction as an equine pregnancy test.

**SUMMARY**

1. The results of the G.-M. test and the A.-Z. reaction for pregnancy diagnosis in mares were compared.

2. Pregnancy diagnosis was carried out on 217 blood samples of inseminated mares. It was later stated that 146 of these samples were from pregnant, and 62 from non-pregnant mares (9 cases could not be taken into consideration).

3. These 208 blood samples were all tested with the Galli-Mainini method; 112 blood samples of pregnant and 57 of non-pregnant mares were also used for A.-Z. reactions.

4. The A.-Z. reaction appeared to be more sensitive for LH than the G.-M. test; moreover the A.-Z. reaction is more reliable and can be used for a longer pregnancy period than the G.-M. test.

5. In the G.-M. test in which *Rana esculenta* was used no positive reactions were obtained before the 43rd day after serving. Between the 43rd and 80th day, 90% of the reactions were positive; between the 80th and 120th day this percentage was 51. After the 120th day no positive reactions were obtained.

6. Taking into consideration that the FSH-signs (increase in size of the ovaries and uterine horns) and the LH-signs (blood spots and corpora lutea) are positive, 100% positive reactions were obtained with the A.-Z. reactions between the 43rd and 80th day after serving and 97% between the 43rd and 190th day after serving. Negative results were obtained in 95% of the tests with bloodsera from non-pregnant mares.

7. Mares’ bloodserum contains a principle, which impedes spermiation.

8. Variability in sensitivity of the test animals, and varying concentrations of the gonadotrophic hormone in pregnant mares’ blood are responsible for an enormous variability in the results of the G.-M. test. In this respect the following facts are important:
a) Frogs used for the first time are 1½ times more sensitive than frogs which had been used previously.

b) The sensitivity of the frogs to gonadotrophins is highest in May/June and lowest in August/September. In the last mentioned months the reaction capacity was reduced to about 50%.

c) The number of previous pregnancies or the age of the mares are of importance: young mares, pregnant for the first time, gave much better results than multiparous older mares.

9. As the G.-M. test is specific, rapid and simple, it is recommended to start equine pregnancy diagnosis with this test and if the result is negative to initiate A.-Z. reactions with the same bloodserum-samples.

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REFERENCES