

# PREGNANCY TEST

## Using Local Male *Rana Tigrina*

BY

V. A. RANE, B.Sc. (Med.), M.D., F.C.P.S.,

*Department of Physiology, Topiwala National Medical College, Bombay.*

In the earliest months of pregnancy there is no single sign or a combination of signs and symptoms to make the diagnosis unequivocal. To overcome these difficulties a number of tests have been devised from time to time. Since the original test described by Aschheim and Zondek in 1928, efforts are constantly being made to find out a simpler, quicker and economical test which would also be absolutely reliable.

*Basis of Biological Tests.* These tests mainly depend upon the presence of the chorionic gonadotrophic hormone which is liberated in large quantities in the blood and urine either by the chorionic villi of the placenta during pregnancy or by the proliferating chorionic tissue in such conditions as hydatidiform mole or chorion-epithelioma. The concentration of the hormone during the different periods of pregnancy as calculated by various authors is shown in the following table.

The amounts of hormone excreted are higher in pre-eclampsia, hypertension and diabetes and very high in hydatidiform mole and chorion epithelioma.

*Female Frog Test.* In 1934 Shapiro and Zwarenstein introduced the fe-

male South African clawed toad *Xenopus lagvis*, as a test animal, the test, with a clear end point of extrusion of macroscopic ova, occurring within 18 hours after the injection, and approaching the reliability of Aschheim and Zondak test.

In 1950 I tried a large number of tests with the female *Rana tigrina*, using known pregnancy urines; the results have been uniformly negative. Bahaduri (1949), Mukherjee and Saha at Calcutta (1952) and others have reported similar negative results in the Indian female Amphibia. Shapiro and Zwarenstein have shown that in *Rana pipiens*, injection of anterior pituitary extract does not cause extrusion of ova. Thus the female frog has no place in pregnancy test because (1) of a uniformly negative result; (2) gonadotrophins have to be extracted from large volumes of urine; and (3) of difficulty in determining the sex of these frogs.

*Male Frog Test.* In 1947 Galli Mainini introduced the male South American toad, *Bufo arenarum* Hensel as a test animal. This apparently marked the beginning of a new era in the biological pregnancy test. Spermatozoa were released on injection of pregnancy urine and this reaction was highly specific, accurate and simple and a rapid means of diagnos-

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TABLE I

*By Prostatic Weight Method in Rats:-*

In the first trimester	20,000 to 50,000 units in 24 hours urine.
In the last two trimesters	4,000 to 11,000 units in 24 hours urine.

*According to Brown and Venning:-*

In the last two trimesters	1,500 to 4,000 units per litre of urine.
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*Smith, Albert and Randall:-*

(Biologically confirmed by a serial assay)

5 days prior to the first expected period .. ..	1,000 to 5,000 units in 24 hours urine.
Between 24th and 40th day after 1st day of L.M.P. ..	10 to 50 units/c.c. of serum.
Between 40th and 90th day after 1st day of L.M.P. ..	High Values.
On 70th day .. ..	Peak excretion.
After 90th day .. ..	2,000 to 15,000 units in 24 hours urine.

ing pregnancy. Wiltberger and Miller (1948) found that the leopard frog *Rana pipiens* responded in a similar manner, there being no error in their 200 tests in the first trimester of pregnancy. Similar results were obtained by Robbins and Parker (1949) and many others. Many reports followed in rapid succession, and most of the workers, besides proving the value of the test have investigated the usefulness of the locally available variety of male amphibia, thus making the test readily available and inexpensive. Mainini by 1948 had collected 27 papers with a total of 3,156 diagnostic and 2,108 control tests. In diagnostic, correct positives varied from 98% to 100%,

the earliest positive result was obtained 4 days after the first missed period. The specificity of the test was shown by the uniformly negative results in all the control tests and negative results with the injection of variety of substances. The credit of using the Indian varieties of these animals goes to Bahaduri (1951). The report of this author and of his collaborator Burdhan (1949) and Mohanti and Pabrai (1950) are based on the results obtained mostly in the male toad *Bufo melanostictus*. Mukherjee and Saha in Calcutta (1952) have used the male frog *Rana tigrina* with good results.

The object of this investigation

was to test the usefulness of the male frog *Rana tigrina* which is freely available in and around Bombay.

*Anatomical and Physiological Considerations of the Test.* The testes are yellowish elongated bodies in the peritoneal cavity attached to the kidney of the same side by a ligament through which pass the collecting tubules. These tubules open in certain glomeruli and carry the sperm. The structure of the testes is similar to that of the higher animals, having seminiferous tubules, interstitial and supporting cells. The kidney tubules, ureter and the bladder form the common excretory channel for both the urine and the sperms. During the breeding season in a particular environment, the male rides and embraces the female. This embrace (amplexus) which lasts for several days, reflexly starts a series of humoral and nervous phenomena, of which the release of the gonadotrophic hormone from the pituitary is the most important. Under the effect of its own gonadotrophin, the female ovulates. The ova, thus released, are fertilised by the sperms of the male, detached by the action of his own gonadotrophin and expelled with the urine. Not only the pituitary gonadotrophin but the chorionic gonadotrophin also gives a similar response. Another substance which was found to give a similar reaction was epinephrine. Follicle-stimulating hormone apparently does not release spermatozoa.

*Mechanism of Release of Spermatozoa.* Giusti and Houssay (1923) demonstrated the intimate relation

between the tests and the anterior pituitary. Houssay and Gonzales (1923), Wolf (1929) and Rugh demonstrated that the anterior pituitary graft or the injection of the anterior pituitary extract released spermatozoa. Robertis, Burgos and Breyer experimented on toads, with injection of anterior pituitary extract and showed on histological examination, that almost all the mature sperms are released due to an intense change (swelling, vacuolation, etc.) in the Sertoli cells. These events occur very rapidly, the changes are obvious within ten minutes and are completed in half an hour. The sperms next pass quickly through the tubules into the ureter, from where they may either be voided or stored temporarily in the cloaca.

In order to test if the local male *Rana tigrina* was similarly responsive or not I injected them with varying doses of (gestyl and pregnyl) chorionic gonadotrophic hormones. The response was very satisfactory. The minimum reaction dose was found to be 10 I.U. and the minimum time for the appearance of sperms was half an hour.

*Spontaneous Spermatogenesis.* Weisman (1948) doubts the reliability of the test. According to him spontaneous spermatogenesis takes place, giving false positive results. In the course of the last three years I have examined samples of urine from over 3,000 male frogs under varying conditions and I have never come across a single instance of spontaneous spermatogenesis. Besides rough handling, different substances

have been injected and none of these have produced any sperms; normal, hypo, or hyper-tonic saline, distilled water, urine of males, urine of menopausal women, clinesterol, oestrin, progestin, male sex hormones, posterior pituitary, adrenaline, vitamins, glucose, etc. etc. It is apparent that in captivity and under laboratory conditions spontaneous spermatogenesis does not take place. This is also proved by the uniformly correct negative results by almost all the workers.

*Determination of sex* is a problem because of the absence of the external genital organs. During the breeding season the sexual differences are striking. As the summer approaches, dimorphism becomes less striking, making it very difficult to be certain of the sex. On dissecting a large series of frogs it is found that except for the loose folds enclosing the black vocal sacs on each side of the throat, which is the only definite sign of the male, all the other signs are not of any use. During summer this pouch generally disappears or is present only in a few big ones. The sex can then be detected by injecting known quantity of pregnancy urine, when sperms appear in the urine of the male frog.

*Material and Method.* Urine can be collected at any time of the day but it must be fresh. The morning sample is preferred. It needs no treatment. Sterilisation of the syringe, the material or the site of the injection is not necessary.

*Site of Injection.* Various sites

for injection have been advocated. The site commonly used is the dorsal lymph sac. In case of *Rana tigrina* the skin being tough and slippery, it is difficult to inject, being inelastic the injecting material leaks out and a higher mortality results due to injury to the lungs. The intraperitoneal and intrapericardial routes carry higher risks.

To overcome these difficulties I have adopted a modified technique as follows:

Holding the frog in the palm of the hand, its mouth is opened by a pair of dissecting forceps. The sublingual mucous membrane is pierced midway between the base and the tip of the jaw with a fine bore needle and the material is injected in the ventral lymph sac which lies underneath the skin (Figs. 1 & 2). The mucous membrane being soft is easy to pierce, being elastic there is not any leakage and has given better results, and no vital structures in the vicinity to cause mortal injury—66% of the frogs injected with the modified technique gave a positive result as compared with those injected in the dorsal lymph sac, 50 frogs being injected in each case.

*Number of Frogs for each Test.* Different authors have injected one or more than one frog for each test. On the basis of the A.Z. test, I have been using as a routine five frogs for each test, two injected with 10 c.c. and three with 5 c.c. of the urine. The necessity of injecting a larger number of frogs can be seen from Table II.

TABLE II

*Necessity of Using a large Number of Frogs.*

Three different series:—

- a) Series of 135 positive in 325 diagnostic tests.
- b) Series with known pregnancy urine, all positive.
- c) Series injected with gonadotrophins, all positive.

Frogs injected with	Total no.	No. positive	No. negative	% positive
a)				
10 c.c. urine ..	208	138	70	66%
5 c.c. urine ..	431	229	202	53%
b)				
10 c.c. urine ..	50	32	18	64%
5 c.c. urine ..	70	34	36	50%
c)				
10 to 100 I.U. gonadotrophins ..	50	35	15	70%
Total ..	809	468	341	58%

From the above results it is apparent that about 42% of the frogs do not react. The results have also brought out the following facts:—

- 20 tests were positive with 10 c.c. while all negative with 5 cc.
- 22 tests were positive with 5 c.c. while all negative with 10 c.c.
- 34 tests were positive with only one out of the 5 injected frogs.
- 18 tests only all the injected frogs gave positive results.

Tests of some of the repeated non-reactors were removed which on microscopic examination did not show the presence of mature sperms. Brody has also encountered frogs which do not react, has frequently noted quantitative difference in their reactivity and has stressed the necessity of injecting more than one frog.

*Seasonal Variation.* Most of the investigators have reported about the seasonal variation in the response and

cyclic spermatogenesis. Having done the test all the year round, no significant seasonal difference in the response was observed in this series. Most probably the reason for obtaining uniform results all the year round is attributable to the existing warmer temperature (75° to 90°F.). The absence of reaction whenever obtained was due to the absence of mature sperms in the testes irrespective of the season.

*Selection of Frogs.* Frogs weighed from 50 grams to 500 grams. The size does not seem to make any difference to the results of the test, big ones respond better with the same amount of urine as is injected into the smaller ones.

*Collection of urine* (Fig. 3) is a very simple process. A small bent glass pipette with a knob at its end is inserted into the cloaca and the urine

easily flows into it. The urine is examined under the low-power of the microscope with the illumination reduced to a minimum. As a routine I always empty the bladder and examine the urine prior to injection. The search for the sperms is sometimes tedious and presents a possible source of error, as sometimes sperms are confused with similar moving bodies in the cloacal flora. The spermatozoa are considerably larger than the human sperms, their heads are long and cigar-shaped, having a long thin tail which has a distinct globular expansion near its middle. Detection of even a single sperm is sufficient to interpret the test as positive. There is no such thing as weakly positive. The number of sperms per field definitely increases in proportion to the concentration of the hormone in the urine. The shortest time in which the sperms have appeared in the urine was half an hour. In the majority of the positive results the sperms appear within three hours, only in two cases the sperms appeared at the fifth hour. If the sperms do not appear by this time the test has always remained negative, even

when the urine has been examined for the next 24 hours.

*Re-use of Frogs.* In a good number of frogs giving positive results, the urine was observed daily and it was found that in the majority the sperms disappeared by 48 hours. The longest time the sperms were detected was five days after the injection and these sperms were generally non-motile and degenerated. Thus the frogs can be re-used after an interval of 10 days.

*Mortality in Frogs.* In over 2,000 frogs injected by now, only 18 have died as a direct result of the test, giving a mortality rate of less than even one per cent and in most of these deaths, the urine was stale and decomposed. As much as 20 c.c. of the urine have been injected at a time without producing harmful effects.

#### *Results of Test.*

When the untraced cases are excluded in this complicated set up, the test has given 95.5% accuracy. On excluding the two cases of false negative in pregnancy which on repetition

*The following are the details of the tests*

TABLE III

Tests for the diagnosis in	Positive	False positive	Positive untraced	Negative	False negative	Negative untraced	Total
Pregnancy ..	88	—	7	121	3	28	247
Abortions ..	17	—	—	15	8	—	40
Vesicular moles	8	—	—	12	—	—	20
Chorion epithelioma	15	—	—	1	2	—	18
Total ..	128	—	7	149	13	28	325

had given positive result, and 7 false negatives in cases of definite abortions (where one knows that the hormones may not be present or present only in low concentration), the accuracy will be 98.5%.

threatened abortion the test was negative, later the bleeding stopped and the pregnancy continued. In two cases of threatened abortions repeated tests remained positive till the time of abortion, hence the test is not

*Classification of Tests on Clinical Findings.*

TABLE IV

Classification of tests on clinical findings	Number of tests	Positive
History of amenorrhoea with size of uterus known ..	149	65
History of amenorrhoea, size of uterus not known ..	51	16
Without history of amenorrhoea, size of uterus known ..	14	3
Abortions: inevitable, threatened, etc. .. ..	40	17
History of scanty or irregular menses .. ..	8	0
Vague history or no history available .. ..	38	23
Vesicular moles and chorion epitheliomas .. ..	25	11
Total .. ..	325	135

*Results according to Duration of Amenorrhoea*

TABLE V

Period of amenorrhoea	Number of tests
In the first week .. ..	2
Between first and second week ..	14
Between second and third week ..	5
Between third and fourth week ..	24
Between first and second month ..	18
Between second and third month ..	4
Over three months .. ..	17
One year .. ..	1
Total .. ..	85

} One case was 5 days, the second 7 days, over their due period

*Comments*

In 3 cases of pregnancy within the first 8 weeks the test was negative, in two of these, the test was repeated two weeks later and gave positive result, the third case could not be repeated. In one case of severe

of value in predicting abortions. It seems worth while to continue with the conservative line of treatment as long as the test remains positive.

In cases of vesicular moles, the test became positive soon after the evacuation of the mole, but in two cases it remained positive for over three

weeks one of which became negative after curettage which showed the presence of vesicles. In all these cases the tests have been repeated at monthly intervals and have remained negative.

*Chorion-Epithelioma.* The following are the results of the two cases of chorion-epithelioma:

(1) In a case admitted for bleeding sometime after the passage of a mole, curettage showed malignant changes. The test done twice, with the urine and also with the C.S.F., gave negative results. A.Z. test done at the Haffkine Institute, both with the urine and C.S.F. was also negative. Skiagram of the chest showed definite metastases. Uterus examined after panhysterectomy was found to be filled with chorionic tissue, and the pathological report was 'malignancy with the predominance of Langhan's cells.' Sieglar, Ravid and Tobin have stated that low and even negative values have been reported to be associated with mole and chorion-epithelioma. It is found that the blood and the urine of the patients afflicted with chorion-epithelioma contains more of F.S.H. than luteinizing hormone, hence the A.Z. test should be more reliable than other tests.

(2) In the second case also admitted for bleeding after the passage of a mole when the test was done for the first time, it was positive one hour after the injection. The biopsy report was 'suggestive of malignant mole' and there was no report of secondaries in the lungs in the skiagram of the chest. The tests were done repeatedly with the results as shown in Table VI.

It is clear from the Table VI that the concentration of the hormone was gradually rising. On this basis the patient was operated, pan-hysterectomy was performed and the uterus showed an invasion of its wall with chorionic tissue and big cysts in the ovaries. The pathological report was 'Chorion-epithelioma'. The tests repeated after the operation were still strongly positive. Skiagram now showed the presence of secondaries in the lungs. On repetition the canon ball shadows were seen to be fast growing. Test done with C.S.F. before, during and after the operation were all negative. The patient was treated without success and she died with severe haemoptysis 40 days after the operation. Brodie and Horwitz have stated that it will almost be providential if the determination of the gonadotrophic hormones in the spinal fluid were diagnostic of a mole or chorion-epithelioma; unfortunately the survey of the literature shows that it is not so. Similar were the findings of Dilworth, May and Hornbuckle, who comment 'that the spinal fluid is a poor substitute for the quantitative Friedman Test.'

*Quantitative tests* were done in the above case. The minimum quantity giving a positive test was found to be 0.05 c.c. of the urine at the last time. On the basis of a minimum of 10 I.U. required to give a positive result in the male frog, it gives a minimum at about 200,000 I.U. per litre of urine in this case. The values calculated in normal pregnancy varied from 20,000 to 40,000 I.U. per litre of urine.



TABLE VI

Amt. inj. in frogs	10 c.c.	10 c.c.	5 c.c.	5 c.c.	5 c.c.	4 c.c.	4 c.c.	1.5 c.c.	1.25 c.c.	1 c.c.	0.5 c.c.	0.5 c.c.	0.4 c.c.	0.2 c.c.	0.1 c.c.	0.1 c.c.	0.05 c.c.
31-1		+	-	-	-												
2-2	-	+	-	-	++												
16-2				+	++	-	+										
26-2			+++	-	+												
6-3	+++	+++	+++	+++	++												
7-3								+	++	+	++	-	+++	++			
14-3			++	-	-												
17-3			+++	+++	+++	+++											
19-3		++	+	++	+												
24-3		-	+++	+++	+												
31-3		+++	+++	+++	+++												
6-4										+	+++			++	++	+++	-
7-4														+++	+	+	-
16-4															++	+	-
20-4														+++		++	+

## Tests with C.S.F.

7-3 „ negative

9-3 „ negative

25-3 „ negative

*Test in Last Trimesters.* A series of 25 tests were done with the urine of pregnant women from 6 months advanced to full term, including five cases from the delivery table. All the 25 samples have given a positive result with the five frog technique. Even if we assume that the urine at this period contains as low as 4,000 I.U., as 10 I.U. are generally sufficient to give a positive result, there is no reason why 10 c.c. of the urine which will contain at least 40 I.U. should not give a positive result. The low percentage of correct positive results, obtained by many authors for the later months of pregnancy, should not be discouraging, since it is not during the second or third trimester that one relies on the pregnancy test.

The diagnostic series includes 50 tests on private patients of Dr. Karande (includes 4 cases of vesicular mole).

*Results*

Correct positive	..	..	—25.
Correct negative	..	..	—23.
False negative	..	..	— 2.
			giving 96% correct results.

One of the two false positives when repeated 6 weeks later was positive.

In 25 positive tests only 42% frogs out of a total of 108 injected reacted.

In 21 of these 50 tests Dr. Karande had done either a cytological or a chemical test.

*Comparative results*

Both positive	..	..	..	..	— 7
Both negative	..	..	..	..	— 6
Frog positive other negative	..	..	..	..	— 6
Frog negative other positive	..	..	..	..	— 2

*Summary and Conclusions*

(1) Biological tests depend upon the presence of the gonadotrophic hormones which appear in the urine immediately after implantation of the ovum. Concentration rises till the end of the first trimester and then falls.

(2) The female frog has no place in the pregnancy test.

(3) Galli Mainini introduced the male toad test. The test is simple and economical and the accuracy is from 98 to 100%.

(4) Locally available male frog *Rana tigrina* reacts equally well.

(5) Anatomical and physiological basis of the test with the mechanism of release of the sperms due to swelling and vacuolation of the sertoli cells is considered.

(6) Spontaneous spermatogenesis does not occur.

(7) Modified technique of injecting in the ventral lymph sac through

the mucous membrane of the mouth is given. Collection of urine from cloaca is simple and easy.

(8) Male carries a black pouch on either side of his throat, without this it is difficult to determine the sex.

(9) Results have proved the neces-

sity of injecting five frogs at a time.

(10) No seasonal variation was encountered in this series.

(11) Presence of even a single sperm is sufficient to interpret the test as positive.

(12) Frogs can be re-used 10 days after the first injection and mortality is very low.

(13) Results of 325 diagnostic tests with 95.5% gross accurate results and on correction give 98.5% correct result.

(14) Positive result was obtained as early as 5 days after the first missed period.

(15) Negative result in one case of chorion-epithelioma with urine and C.S.F. Rising titre in the 2nd case of chorion-epithelioma was diagnostic.

(16) Test can also be used as a quantitative test.

(17) Uniformly positive results in known pregnancies in the last trimester.

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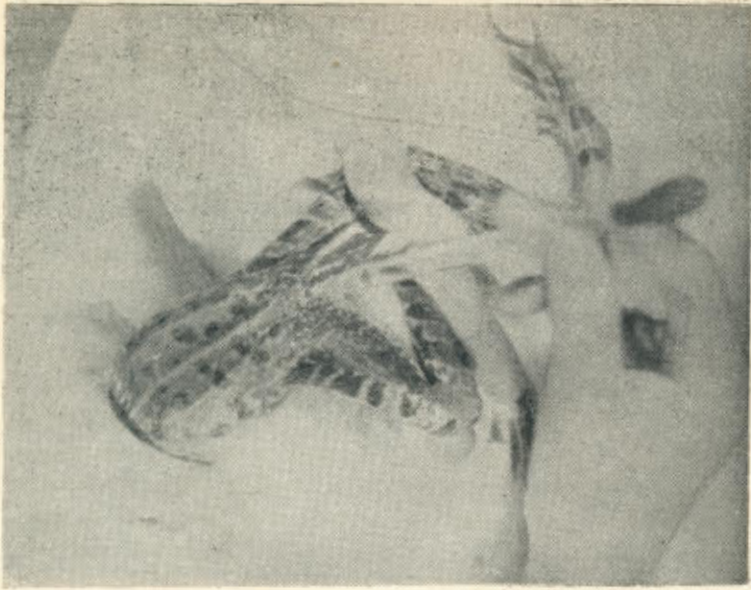


Fig. 3.: The method of collecting the urine from the cloaca.