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The Frog test in the diagnosis of pregnancy

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THE FROG TEST IN THE DIAGNOSIS OF PREGNANCY

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INTRODUCTION

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The search for a satisfactory test for the diagnosis of pregnancy has continued through the centuries. Henriksen (1) reviewed the major resulting contributions, most satisfactory of which have been biologic tests, the Ascheim-Zondek using immature female rats and mice and the Friedman which employs mature female rabbits. The basis for these procedures is the action of chorionic gonadotropin (present in pregnancy urine) producing hyperemia, maturation and rupture of ovarian follicles in the test animal.

The use of amphibia in determination of pregnancy was introduced in 1934 by Balleray (2), who observed that the female South African clawed toad, Xenopus laevis, on injection of pregnancy urine, releases large quantities of eggs within 6 to 15 hours. In 1947 Galli-Mainini (3) reported that the male toad, responds to urinary chorionic gonadotropin by the release of spermatozoa, in as short a time as one hour following injection. Shortly thereafter Robbins and Parker (4) and Wiltberger and Miller (5) published the use of the common North American grass frog, Ranapipiens. Subsequently the world literature has been replete with articles, reporting mechanism of action, use of differing species of amphibia, varying techniques, accuracy percentages, analysis of false responses, differential diagnosis of pathologic conditions and comparative studies with parallel use of other biologic tests.

This paper reviews the more important contributions, presents the

author's personal series, and attempts an analysis of the errors in diagnosis. Suggestions for avenues of further study are offered and a method of performing the frog test *, designed to achieve the maximum reliability, is presented.

Partial reviews of the literature may be found in publications by Galli-Mainini (6) and Thorborg (7).

* Through common usage in this country, the term "frog test" seems to have acquired an all-inclusive acceptance, regardless of the species of amphibian used. In this sense it will be used in this paper.

MATERIAL AND METHODS

Basically the technique of performing the frog test is simple. The substance to be tested is injected into the dorsal or lateral lymph sac, or the injection may be intraperitoneal. Subsequently, the cloacal secretion is examined for the presence of spermatozoa, which finding comprises a positive response. Many factors, however, concerning the test animal, injection technique and the test substance must be considered.

A. THE TEST ANIMAL

1. SPECIES

A great variety of amphibian species have been used in the frog test for pregnancy diagnosis. Galli-Mainini (3) and Earle (8) used the large male Bufo arenarum and Bufo marinus, respectively. The first North American investigators (4, 5) obtained good results with the leopard frog Rana pipiens. Later, McCallin and Whitehead (9, 10) and Soucy (11) introduced several smaller species of toads. Thorborg (7) reviewed the literature in 1950 and reported the use of a large number of species in many countries. This author considers the male Xenopus laevis unsuitable because of the many false positive and spontaneously positive reactions which have been observed.

2. SEASONAL VARIATIONS

Soucy (11) and Samson (12) called attention in 1950 to the existence of a seasonal variation in the response of Rana pipiens to chorionic hormone stimulation. They noticed that during the summer months the

accuracy of the frog test was much diminished due to an increased incidence of false negative reactions. At the same time Soucy found that three species of toads, Bufo cognatus, Bufo americanus and Bufo terrestris gave consistently correct results with the same specimens which were producing false negatives in the leopard frog. Holyoke and Hoag (13) through studies with preparations of hormones of known strength, found that the male Rana pipiens undergoes a 10-fold decrease in reactivity to chorionic gonadotropin during the summer months. Jeffree (14) feels that the toad is less sensitive during the fall and winter months. Thorborg and Hansen (15) found that Bufo bufo requires 2-3 times as much chorionic gonadotropin to produce a response in August as it does in March. Reinhart and co-workers (16) noted that the male Rana clamitans (common North American water frog) reacts just as well to hormone stimulation during the summer as does Rana pipiens during the winter.

3. INDIVIDUAL CHARACTERISTICS

The selection of the test animal is based primarily on sex. The male frog is characterized by the presence of prominent clasping digits and pads on the forelimbs, and vocal air sacs on the neck. The male toad is recognized by its croak and the dark pigmentation on its throat.

Weight of the animal is considered important by Reinhart et al (16) who found that frogs weighing more than 41 gm failed to respond to 0.8 mg. of a crude gonadotropic powder, whereas test animals of 30-40 gm. did respond. However, Haskins and Sherman (17) used frogs

weighing 28-50 gm. in their technique for quantitative bioassay of chorionic gonadotropin. Galli-Mainini (3) used toads weighing 100 or more grams while those used by Earle (8) were 150-200 grams. These two investigators injected 10 ml. of urine while the usual amount initially used with smaller amphibia was 5 ml. Bieniarz (18) used a quantity of urine equivalent to 1/20 of the weight of the test animal, while Hodgson (19) recently reported using one ml. of urine per 10 grams of body weight.

4. REPEATED USE OF ANIMALS

Greenblatt et al (20), McCallin and Whitehead (10) and Holyoke and Hoag (13) have observed that repeated use of frogs and toads, which have given positive responses, apparently results in decreased sensitivity to the stimulating hormone. That this refractoriness is due to depletion of the spermatozoa has been shown histologically by Bieniarz (18). A further complication of the use of previously positive reactors is the presence of residual sperm. Thorborg (21) found that a high percentage of positive responding toads stored in the cold continued to excrete spermatozoa even after 24 days. If left at room temperature, all of the animals were negative after 8 days. We have observed on several occasions, that although the initial check of a used positive refrigerated frog was negative, examinations subsequent to exposure to room temperature for a short period of time revealed the presence of spermatozoa in the cloacal secretion. For these reasons we disgard all positive test animals.

Hodgson (19) has stressed the importance of the initial check of all

test animals for spermatozoa. Three times while doing 150 tests, she found spermatozoa in unused frogs. Of some 800 odd tests we have performed spermatozoa have been found in fresh test animals only 4 or 5 times. That such a possibility exists, however, warrants a careful preliminary check of all test animals.

5. TEMPERATURE

Another factor which has been given some consideration is the matter of temperature. Reinhart and his co-workers (16) claim that if the amphibia are kept at 15-22 degrees Centigrade during the test, an increased number of spermatozoa will be released and more reliable results obtained. Most workers have maintained the animals at room temperature with apparently equally good results.

6. STORAGE

The laboratory storage of amphibia is a relatively simple matter. Hodgson and Taguchi (22) and Glitz and Miller (23) feel that refrigeration is an essential factor for obtaining a valid test. The latter authors state that dehydrated animals may give a false positive reaction on injection with large quantities of liquid. The animals may be kept in large groups or may be separated into individual containers. The major measure is to provide sufficient moisture. This may be done by adding 1/8 to 1/4 inch of water to the container. More than this is not necessary. This water should be changed at least every other day to prevent the accumulation of waste products which may prove toxic to the animals. Forman (24) keeps his toads

in the refrigerator on moist paper in individual containers.

We prefer the method of Gardner and Harris (25) of storing frogs in a covered enamel refrigerator pan with peat moss covering the bottom of the pan and just enough water to thoroughly wet the moss. We store our unused frogs in this manner and used negative animals are replaced in the refrigerator in individual covered glass containers with just sufficient water to moisten the inside of the glass. The pans are cleaned every 2 weeks to receive a fresh group of frogs. No food is required for the animals as their metabolism is depressed under refrigeration. Lehman (26) reports using chloromycitin palmitate to combat "red leg" in frogs. We have encountered no difficulty with this infection using the above described storage conditions.

B. TECHNIQUE

The original technique of injecting unaltered urine was soon found to be not entirely satisfactory. Numerous investigators (9, 13, 20, 27) have commented on the death of injected frogs, apparently due to the presence of toxic urinary excretory products in the specimen. This not only invalidated the tests but required obtaining a second specimen with considerable time delay. Another disadvantage of this method, is a relatively high incidence of false negative responses. There are probably two factors responsible for this. First, the chorionic gonadotropin titer is low during the first month of pregnancy and again during the last two trimesters (17, 20, 28, 29, 30). Bromberg et al (31) encountered an incidence of 74.3% false negatives

in tests run prior to the tenth day past the expected menstrual period, while there were 34.6% false negatives after the fourth month of pregnancy. Gardner and Harris (25) noted an incidence of 53% false negatives during the last two trimesters.

The second factor responsible for false negatives is the seasonal variation in frog response as discussed above. Several workers have used less than 5 ml. of urine and have thus avoided toxicity to the test animals, but in so doing have apparently increased their incidence of false negatives through lowering the amount of injected hormone (25, 31, 32).

Cutler (33) in 1949, and Samson (12) in 1950, in order to circumvent these disadvantages, suggested the use of the Scott (34) concentration method (Appendix A), whereby the toxic elements are eliminated and the quantity of hormone available for injection in small volume is greatly increased. Loraine (28) and Dekanski (35) found this procedure, utilizing kaolin (aluminum silicate) to adsorb urinary gonadotropins, to be an efficient method providing yields as high or higher than those obtained by other methods. Cutler (33) stated that this method might lead to the production of false positive reactions in menopausal specimens with high titers of follicle stimulating hormone (FSH). However, Bradbury and co-workers (36) failed repeatedly to recover pituitary gonadotropin by this method and they conclude that the pituitary gonadotropin is easily and rapidly destroyed

by the sodium hydroxide used in the procedure.

Reinhart et al (16) prefer the use of the cold acetone method (Appendix B) in extraction of urinary gonadotropins. In their hands this method has resulted in a higher accuracy than has the kaolin technique. Levey and Putnam (37) have introduced the use of Amberlite XI-96, an ion exchange resin (Appendix C). While this procedure de-toxifies the urine, it is not a concentration technique and therefore does not solve the problem of false negative responses.

The use of blood serum was reported by Haskins and Sherman (17, 29). Each of two frogs is injected intraperitoneally with 3 ml. of serum from centrifuged blood. The rationale behind this procedure is twofold: First, the serum is apparently devoid of the toxicity associated with urine; and second, the chorionic gonadotropin titer is 10-15 times higher in serum than in urine (29). Hodgson (19) has adopted the serum method and claims excellent results.

Regardless of the test substance used (urine or serum), or the method of injection, examination of the cloacal secretion is made over a period of 2 hours following injection. If unaltered urine is used, it is probably best to continue examination for a total of 3-4 hours. Galli-Mainini (6) reported that 100% of the reactions would occur within a space of 3 hours following injection. Robbins and Parker (38) state that if an adequate stimulating

dose is injected (using Rana pipiens) spermatozoa will appear in the cloacal fluid 15-20 minutes later and the reaction becomes maximal in 1-2 minutes. We have found that in a high percentage of cases spermatozoa appear 10 minutes following injection.

PRODUCTION OF THE GAMETOKINETIC RESPONSE

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A. SUBSTANCES PRODUCING RESPONSE

Houssay, et al (39) in 1929 reported that injection of anterior pituitary extracts would produce very rapid release of spermatozoa in the male toad. As mentioned above, Galli-Mainini (3) reported, in 1947, a similar response to the injection of pregnancy urine. In 1949 Li and Chang (40), Hinglais and Hinglais (41) and Robbins and Parker (42) published evidence that Adrenalin causes gametokinesis (release of spermatozoa) in several species of male frogs, whereas male toads of the Bufo genus are not sensitive to this substance.

Clark et al (43) in 1951 reported the response of the male Rana pipiens to levo-epinephrine and levo-arterenol. Earlier the same workers, Greenblatt et al (44) used various commercial hormone preparations, including pituitary extracts, purified FSH, combinations of anterior pituitary and chorionic gonadotropin, pregnant mare's serum, prolactin and interstitial cell stimulating hormone, obtaining the release of spermatozoa with each substance. Sulman (45) in 1951 produced the gametokinetic response with adrenal cortex extracts, but felt this was probably due to contamination with medullary epinephrine. Robbins and Parker (46) also obtained the response, although inconstantly, to aqueous adrenal extract and to dehydroisoandrosterone from the adrenal gland, as well as to aqueous Androstine A from the testes. In their study, alcohol-

precipitated menopausal urine, pituitary and urine gonadotropins and epinephrine invariably caused the release of spermatozoa, whereas estrogens and progesterone were invariably negative in action.

Other substances which have been shown to be gametokinetically inactive are prostigmine, histamine phosphate, dexedrine, neosynephrine and ACTH (44). Li and Chang (40) obtained no response with ephedrine. Robbins and Parker (42) noted a seasonal variation in the Rana pipiens reaction to Adrenalin, no response being obtained during the summer months of June, July and August. Xenopus laevis, however, reacted all year around to this substance.

B. MECHANISM OF ACTION

1. ANATOMY

The kidneys of the male frog consist of a pair of flattened, elongated bodies, lying in the retroperitoneal space just anterior to the cloaca. On the ventral surfaces of these structures are located a pair of small, bean-shaped testes. Connecting the two organs are slender ducts, the vasa efferentia, through which spermatozoa pass from the testes into the kidney tubules. (Note: Thorborg has demonstrated that in Xenopus laevis and Bufo bufo spermatozoa reach the kidneys not only via the vasa efferentia but also by a direct extracanalicular passage whereby the spermatozoa penetrate the capsules of both testes and kidneys, passing through the lymph and blood sinuses surrounding these structures. This apparently represents a more primitive mechanism than that found in Rana esculenta where the tubules are further developed

and no extracanalicular passage of sperm was observed.)

From the kidneys spermatozoa and urine are transported by a common duct into the cloaca. A thin-walled bladder lies ventral to the cloaca, its opening being opposite the ureteral apertures. The urine and sperm thus enter first into the cloaca, then are either expelled to the outside or passed into the bladder for temporary storage.

In the amphibian the adrenal gland is also located on the ventral surface of the kidney as a yellow longitudinal strip in intimate contact with the kidney structure.

2. PHYSIOLOGY

Galli-Mainini (48) in 1948 stated that "gonadotropic administration stimulates the interstitial cells and spermatogenesis and produces detachment of spermatozoa from the Sertoli cells." Whether this is a direct action on testicular tissue or an indirect stimulation via the pituitary and/or adrenal mechanisms is not yet clearly determined. The positive response to both adrenal and pituitary factors implies the possible involvement of these glands.

Clark and co-workers (43) found that they could block the male frog response with several adrenolytic drugs. Of those used Benodrine hydrochloride was the most effective. The adrenolytic agents were administered 30-60 seconds prior to injections with chorionic gonadotropin, anterior pituitary hormone, human pregnancy urine, levo-epinephrine, levo-arterenol and epinephrine hydrochloride.

They felt that this evidence suggests an epinephrine activation of an adrenotropic receptor in the sperm ejaculatory apparatus of the male frog. Sulman (45) attempted adrenalectomy in Rana pipiens, Bufo viridis and Hyla arborea by cauterization of the adrenal gland. Following this procedure he found no interference with the gameto-kinetic response to chorionic gonadotropin and therefore concluded that no hormonal relationship exists between epinephrine ejaculation and gonadotropin ejaculation. Robbins and Parker (38) ruled out the possibility of neural pathway involvement by destruction of the central nervous system and transsection of the spinal cord with no inhibition of response resulting. Likewise, hypophysectomy failed to inhibit the release of spermatozoa. By direct injection into either testis or adrenal a response was obtained with 1/4 the quantity of chorionic gonadotropin required subcutaneously to produce a response. These workers feel that the adrenalin response is mediated through the production of cortical steroids which probably act on the testis secondarily, while the gonadotropin response is due to direct action of the hormone on the testis. The evidence thus points to two mechanisms which are not necessarily related. Further support to the direct action of gonadotropins on the testis is the work of Burgos and Mancini (49). These investigators cultivated testicular tissue from Bufo arenarum "in vitro" and were able to elicit complete release of spermatozoa by the addition of 0.5 I.U. of chorionic gonadotropin.

C. CHORIONIC GONADOTROPIC HORMONE LEVELS

1. NORMAL PREGNANCY

Loraine (28) in 1950 published her estimations of the varying levels of urinary chorionic gonadotropin in normal pregnancy. Using the first day of the last menstrual period as day "0", she found that from the 40th to the 80th days, 20,000 to 40,000 I.U. were eliminated in the urine over a 24 hour period. The titer drops rapidly and from the 100th day to term the normal range is 4,000 to 11,000 I.U. per 24 hour specimen. By the third postpartum day the hormone level was found to have dropped below 1,000 I.U. per 24 hours. The quantitative studies in this series were based on the prostate weight increase in the male rat. Smith and co-workers (30) using the rat uterus and ovary response to chorionic gonadotropin, found that from day 24-40 1,000 to 5,000 I.U. appear in the 24 hour specimen of urine. Immediately after day 40 there is a sharp increase in the level reaching a maximum of up to 450,000 I.U. at day 70. Serum levels during the 24-40 day period were estimated to be 10-50 I.U. per m.l. of serum. These workers observed that the most frequent time for the appearance of chorionic gonadotropin was on the 23rd day of the cycle or approximately 5 days before the first missed menstrual period.

Haskins and Sherman (17) in 1949 published a quantitative bioassay method for chorionic gonadotropin determination, using the male frog, Rana pipiens. They established a curve relating the time required for the gametokinetic response to the dosage of hormone. In 1952 these

workers (50) reported a normal range of 10-120 I.U. of chorionic gonadotropin per ml. of serum with the peak at the 62nd day, a rapid decline in titer to the 154th day, followed by a slight continued rise until the termination of pregnancy.

From the above data it will be noted that the minimum 24 hour urinary excretion of chorionic gonadotropin during normal pregnancy is in the neighborhood of 1,000 I.U. Assuming the average 24 hour excretion of urine to be 1500 ml. we can expect to find a minimum of 0.67 I.U. of hormone per ml. of urine. Likewise we note from these workers that the minimum titer of chorionic hormone in the serum is 10 I.U. per ml. These data are summarized in Table I.

Table I. Normal Pregnancy Levels of Chorionic Gonadotropin in Urine and Serum (International Units)

Author	Day of Pregnancy Cycle			
	24 - 40	40 - 90	90 - 120	100 to Term
URINE (per 24 hours)				
Smith (30)	1000 - 5000	up to 450,000	2000 - 15,000	---
Loraine (28)		20,000 - 40,000		4,000 - 11,000
Minimum I.U. per ml. urine*	0.66	13.6 I.U.		2.7 I.U.

* Assuming average daily urine output of 1500 ml.

SERUM (per ml.)				
Smith (30)	10 - 50	Variable	10 - 100	
Haskins (50)	10	to	120	
Minimum I.U. per ml. serum	10			

The male toad has been reported to respond to minimum quantities of chorionic gonadotropin varying from 2 I.U. to 12.5 I.U. (15, 31). Haskins and Sherman (17) assigned to the frog unit a value of 35 I.U. of chorionic hormone, on the basis that 100% of Rana pipiens would respond to this dosage. Samson (12) found that this animal would respond to between 2 and 5 I.U. during the fall, winter and spring, but required 10-20 I.U. of chorionic gonadotropin in the summer months. Holyoke and Hoag (13) found that Rana pipiens would not react to as much as 80 I.U. during the month of August, whereas in October 100% of these animals gave a positive response to 20 I.U. Reinhart et al (16) report that Rana clamitans responds to 0.8 mg of chorionic gonadotropin (8.0 I.U.) in July.

Correlating the above data, we may roughly conclude, providing either the toad or Rana clamitans is substituted for Rana pipiens from May through September, that the frog test will be sensitive to 35 I.U. of chorionic gonadotropin the year around. To assure the presence of 35 I.U. in the injectate, a minimum of the equivalent of 50 ml. of urine* should be used during the 24-40 day period of early pregnancy. During the same period 3.5 ml. of serum should be adequate to elicite a positive response in the presence of normal pregnancy. The remainder of the first trimester a minimum of the equivalent of 3 ml. of urine should be used and during the last two trimester the equivalent of 20 ml. of urine should produce a positive response in the presence of a normal pregnancy.

* It is generally accepted that the specific gravity of the urine specimen be at least 1.010.

2. PATHOLOGIC CONDITIONS

The level of chorionic gonadotropin is variable in abnormal pregnancy such as ectopic pregnancy, abortion, diabetes and toxemia, and in such conditions as hydatidiform mole and chorionepithelioma, which must be considered in differential diagnosis. The results of the frog test must be evaluated accordingly.

Bromberg and co-workers (31) reported an incidence of 88% negative responses in conditions of ruptured ectopic pregnancy. Haskins and Sherman (29) found that 50% of the proven ectopic pregnancies in their series were characterized by negative frog tests, while Holyoke and Hoag (13) obtained a 43% incidence of negatives. In the series reported in this paper 4 ectopic pregnancies were encountered and a negative test was obtained in one case. The remaining three cases were weakly positive.

The titer of chorionic gonadotropin decreases in pending abortion (51) and if it persistently falls between the fourth and twelfth week of pregnancy, inevitable abortion is probable according to Bieniarz (18) and Hartleb (52). Bieniarz states that a persistent negative after a falling intensity indicates the death of the fetus.

An increase in chorionic hormone titer has been noted by Loraine (28) to occur during the last two trimesters of pregnancy in many cases of diabetes, pre-eclamptic toxemias and essential hypertension. Haskins and Sherman (50) in their quantitative studies using the

frog, also reported increased chorionic gonadotropin titer in eclampsia as well as in a case of enzygotic twins.

Chorionic gonadotropin is produced ordinarily in greatly increased amounts in cases of hydatidiform mole and chorionepithelioma. However, Thompson et al (53), Acosta-Sison (54) and Mueller and Lapp (55) have reported that early cases of chorionepithelioma may be characterized by negative biologic tests. Lehman (26) reports a case of chorionepithelioma in which a positive test was obtained using 0.001 ml. of urine and Haskins and Sherman (50) in a case of this type report a 1,620% increase over the average level of chorionic gonadotropic hormone for normal pregnancy. The same authors found a 300 to 6,500% greater chorionic hormone titer in hydatidiform mole than in normal pregnancy. With hydatidiform mole, Hodgson (19) obtained a positive test in 1/700 dilution and Earle (8) did so with 0.05 ml. of urine in dilution. Hartleb (52) feels that a positive test with 0.02 ml. of urine is suggestive of a mole.

Lehman (26) noted that tests remained positive for several weeks following the removal or passing of a mole. Minet and co-workers (56) suggest that tests should be run weekly after the expulsion of a mole and further state that an increase of gonadotropin level after an initial fall, or a level higher after one month than that at the time of explusion, is definite proof of complications, such as recurrence or development of a chorionepithelioma.

3. QUANTITATIVE STUDIES

That quantitative studies may be of definite value on differential diagnosis is readily discerned from the above data. Hartleb (52) describes a rough quantitative test using 10, 1-2, 0.7, 0.5, 0.2 and 0.02 ml. of urine. He finds that the first 5 stages are usually positive in normal pregnancy while a decrease in the number of positive stages or repeated negative responses in the 4th and 5th stages are suggestive of inevitable abortion. As noted above this investigator feels that a positive response with 0.02 ml. suggests a possible mole. This type of test is practical, but because of species and seasonal variations in response, the interpretation must depend upon experience with the particular species used.

The quantitative bioassay method for chorionic gonadotropin determination developed by Haskins and Sherman (17, 50) based on the relationship between the dose of hormone and the time required for response may be used if precise knowledge is desired. In order to accommodate this method to the seasonal variation of the Rana pipiens frog standardized curves are made frequently throughout any prolonged study.

ACCURACY

ACCURACY

In order to more effectively evaluate the varied accuracy percentages reported with the use of the frog test for pregnancy, the reports are placed into four categories (Table II), two of which are concerned with the use of unaltered urine, the third with concentrated urine and the fourth with the use of serum. The accuracy achieved with the frog (primarily Rana pipiens), using unaltered urine is 87.5% as compared with 95% for the toad, 97.6% with concentrated urine (again primarily Rana pipiens) and 97.3% using serum.

Table I. Accuracy Percentages of a Random Selection of Frog Test Reports Separated into Four Groups on the Basis of Technique.

A..Using Unaltered Urine	Injectate	No. of Tests	False Positives	False Negatives	Total Errors	Perdent Accuracy
Group I - Frogs						
Rana esculenta (18)	1/20th wt of frog	92	0	5	5	94.6%
Rana pipiens (63)	4.0 ml.	108	0	21	21	81.0%
Rana pipiens (25)	2.5 ml. + 2.5 ml.	256	0	112	112	56.3%
Rana pipiens (20)	5.0 ml.	323	2	8	10	96.9%
Rana pipiens (59)	5.0 ml.	300	1	12	13	96.0%
Rana pipiens (5)	5.0 ml.	200	0	0	0	100.0%
Total -		1279			161	87.5%
Group II - Toads						
Bufo bufo (65)	3 - 5 ml.	815	3	84	87	89.4%
Bufo marinus (8)	10.0 ml.	850	-	40	40	95.3%
Bufo americanus (24)	2.5 to 3.0 ml.	268	0	5	5	97.9%
Bufo arenarum (6)	10.0 ml.	3156	0	60	60	98.2%
Bufo arenarum (14)	2.0 ml.	205	-	-	76	63.0%
Total -		5254			268	95.0%

Table I. (cont'd)

B. Using Concentrated Urine	Injectate (urine equivalent)	No. of Tests	False Positives	False Negatives	Total Errors	Percent Accuracy
Group III - Frogs						
Rana pipiens (33)	20.0 ml.	200	0	2	2	99.0%
Rana pipiens (64)	50.0 ml.	114	0	11	11	93.4%
Rana pipiens (57)	30.0 ml. 60.0 ml.	207	6	15	21	90.0%
Rana pipiens (22)	20.0 ml.	87	0	2	2	97.7%
Rana pipiens and Rana clamita ns (16)	10.0 ml. 5.0 ml.	1191	0	3	3	99.0%
	Total -	1619			39	97.6%
C. Using Blood Serum						
Group IV - Frogs						
Rana pipiens (19)	1.0 ml. per 10.0 g. wt.	100	0	1	1	99.0%
Rana pipiens (29)	3.0 ml.	800	-	-	23	97.0%
	Total -	900			24	97.3%

ANALYSIS OF FALSE RESPONSES

ANALYSIS OF FALSE RESPONSES

Consideration has been given in the section on materials and methods, to the factors of species and seasonal variation and the repeated use of the test animals as contributing to false responses. Also mentioned has been the matter of using unaltered urine in quantities which contain insufficient hormone to produce the gametokinetic response. Thus the factors in regard to the test animal and to the technique of performing the test have been covered. A third factor, and one which is not as readily correctible, is the patient. Speaking generally, the factors of test animal and technique are primarily concerned with false negative responses. The patient, on the other hand, contributes mainly to the false positive responses.

Ovarian pathology has been incriminated several times in non-pregnant patients whose urine has produced a positive frog test. Lehman (26) reports obtaining twelve positive tests over a period of sixteen weeks on a patient who subsequently had an ovarian cyst removed. Following surgery the test became negative. Bromberg et al (31) performed frog tests on four patients with corpus luteum cysts. Two of these tests were positive and two gave negative responses. Mause (57) had a false positive reaction with a specimen from a patient with a large paraovarian cyst, and Greenblatt et al (20) report positive frog tests in one case of salpingitis with an ovarian abscess and also in a case of multiple mucous cyst

adenomata of the ovary. These investigators suggest the possible destruction of ovarian tissue leading to increased production of FSH which then results in a false positive test.

The high titer of FSH in menopausal urine has also been suggested by Cutler (33) as a possible source for false positive reactions. Hodgson (19) reports two cases of weak false positives in patients aged 44 and 52 years that may fit into this category also. Thorborg (15), however, on the basis of failure of toads to respond to as much as 20 mg FSH preparation, states that it is "unlikely that menopausal urine will cause false positive spermiation reactions." Phillips (58) states that he has obtained positive responses consistently with 25 I.U. of FSH (Gonadophysin). Clearly, this area of possible false positives needs further investigation.

Another source of false positive reactions which remains obscure lies in patients who exhibit jaundice. Robbins and Parker (59) first reported, in 1949, false positive tests on two patients with icterus. Sobel and Edelman (60) obtained positive frog tests in nine out of twenty-one patients with jaundice. The positive reactors included five males and four females. The substance responsible for these false positives has not yet been identified, but is described as being apparently labile as the activity disappeared if the substance were kept on kaolin for several days. Lehman (26) ran tests on fourteen patients with hepatitis and three with obstructive jaundice but failed to get a positive response. This too represents a field for further investigation. Possibly the answer lies in the

presence of sufficient damage to the liver to render it unable to de-toxify pituitary gonadotropins.

Elsewhere in this paper the gametokinetic response to Adrenalin is discussed. Greenblatt et al (44) have reported the case of a patient with myxedema to whom an infusion of 1.5 mg. of epinephrine was given as a test of pituitary insufficiency. Fifteen minutes later a urine specimen was obtained which produced a positive frog test. This suggests the possibility of obtaining a positive test on urine in the presence of a pheochromocytoma. Van Euler (61) has stated that in this condition the daily urinary excretion of Adrenalin and noradrenalin increased from normal values of 10 and 30 micrograms to figures 10-50 times as high.

Other false positives that have been reported include one 22 year old single female with a large fibroid (19), one patient six months postpartum, not yet menstruating and several possible early abortions (56, 57).

AUTHOR'S SERIES

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During the period from September 1950 to June 1953, the author performed 602 frog tests for pregnancy diagnosis. Of this number 387 cases have had complete follow-ups. 212 tests gave a positive response, 172 were negative. The break-down of these tests is listed in Table III. Negative tests in cases of abortion and ectopic pregnancy were not considered erroneous.

A. MATERIALS AND METHODS

Rana pipiens frogs were used exclusively during the first year, including the summer months of 1951. It was noticed during June, July, August and September of that year that the response to pregnancy urine was very weak in spite of the fact that the kaolin extraction was used throughout (see Appendix A). Thereafter, the Rana clamitans frogs were used during the summer months, beginning about May 15 and continuing until the end of September. Occasionally, if the clamitans frogs were not available, Bufo americanus was substituted. All of the test animals were procured from two biological supply houses in Wisconsin and Minnesota. The method of storing the animals has been described under the major section on materials and methods above.

Initially, both positive and negative frogs were reused following a rest of at least one week. It was soon noticed, however, that reusing positively reacting frogs was unsatisfactory and this practice was discontinued. We have continued to re-use negatively responding

Table III Breakdown of 387 Tests for the Diagnosis of Pregnancy

Proved Positive Tests		
Delivered at term	143	
Autopsy, 4 months gestation	1	
Subsequent abortion	52	
Ectopic pregnancy	3	
Chorionepithelioma	1	
Hydatidiform mole	1	
		202
Proved Negative Tests		
Clinically not pregnant	155	
Abortions	7	
Intrauterine fetal death	1	
Ectopic pregnancy	1	
		164
False Positive Tests	10	
False Negative Tests	8	
		18
Total Tests		387

frogs one or two times up to the present time, but always in conjunction with the use of one fresh frog.

The only request made in regard to the urine specimen was that it should be at least 100 ml. in quantity. However, all specimens were accepted, providing they were at least 20 ml. in quantity. No consideration was given to the time of day collected nor to the specific gravity.

The specimens were extracted, as indicated above, by a modification of the Scott technique (Appendix A). During this procedure the test animals were removed from the refrigerator, placed in covered clean glass containers and checked for the possible presence of spermatozoa prior to injection.

The injection technique is as follows using a 5 ml. Luer-Lok syringe and a 1 inch 25 gauge needle. The frog is grasped in one hand with the fingers wrapped around the hind legs and the thumb pressing on the back of the thighs so that these extremities are held in complete extension. In this manner the frog is very easily controlled. The needle is inserted just through the skin of the thigh about 1 cm distal to the torso. The needle is then directed cephalad and mediad to pass through the posterior boundary of the dorsal lymph sac. One half of the total extract is injected into each of two frogs. Swelling of the entire dorsal lymph sac is noticed as the injectate is introduced.

The animals are replaced into the glass containers and routinely examined at intervals of ½ hour for a period of 2 hours. One group of 52 positive reactors was examined at 10 or 15 minutes as well as at 30 minutes. 31 of the tests were positive within the 15 minute period following injection. 7 additional tests had responded by 30 minutes and 14 did not respond until after 30 minutes. In no cases were positives observed to occur after 2 hours, and in no instance has a positive been observed in less than 10 minutes following injection.

Examination of the frog is carried out in the following manner. The thumb and forefinger of each hand grasps a hind limb and folds it up along the lateral body wall. The cloacal orifice is then placed against a glass slide and almost 100% of the time a drop of secretion will be extruded. In the case of the toad, it is necessary to insert a pipet into the cloaca and thereby remove a drop of secretion. The secretion is examined under the microscope with reduced light for the presence of motile, banana-shaped dark rods. Occasionally there will be no motility exhibited.

The response is roughly graded from one plus to four plus. A one plus represents a response characterized by the presence of a maximum of 5 sperm per low power field. This is considered a very weak response. A low power field containing from 6-30 spermatozoa would be rated a two plus or a relatively weak response. A grading of three plus indicates that the low power field is fairly well covered with spermatozoa and a four plus grading is conferred on the response characterized by actual crowding of the microscopic field.

A three plus or four plus response is to be expected at any stage of pregnancy following the 40th day of the menstrual cycle. In our series 15 positive tests were performed prior to the 40th menstrual cycle day. Of these 12 or 80% were characterized by the presence of a three plus response. Galli Mainini (6) has reported correct positive results in 33 patients with amenorrhea of 4-10 days duration. Hodgson (19) states that a positive frog test may result as early as the 30th day of the menstrual cycle. Two well-documented cases in our series resulted in positive responses on the 26th and 31st cycle days respectively.

B. ACCURACY

The over-all accuracy of this series is summarized in Table IV. 18 false responses occurred in 387 tests; the accuracy therefore is 95%. 5 of the 18 false responses occurred during the summer months of 1951 when the more refractory Rana pipiens were being used. If we temporarily eliminate from the series all of the tests performed during this period of June through September, 1951, we find that of a total of 339 tests there were 13 false responses or an accuracy of 96.2%, which figure presents a more valid picture of the expected results as the test is currently being performed.

Table IV. Over-all Accuracy of Results

Period	Total No. of Tests	False Positives	False Negatives	Percent Accuracy
Sept., 1950 to June, 1953	387	10	8	95%
Same, eliminating June, 1951, to October, 1951*	339	10	3	96.2%

* Period when Rana pipiens used during summer months.

A comparison of the tests performed during the summer of 1951 using Rana pipiens, with those performed during the summer of 1952 when Rana clamitans were employed, is shown in Table V. 5 false negatives were obtained in 48 tests using Rana pipiens. The following year only one false negative occurred using Rana clamitans. One false positive was also noted with the latter group. The accuracy therefore using Rana clamitans during the summer months was 95.7% as compared with 89.6% for the Rana pipiens during a similar period. This comparison bears out the advisibility of substituting a more sensitive animal for Rana pipiens during the summer, in addition to taking the precaution of concentrating the urine specimen.

Table V. Comparison of Results in Summer Months, 1951 and 1952 using Two Species

Year and Test animal	Total No. of Tests	False Positives	False Negatives	Total Errors	Percent Accuracy
1951 Rana pipiens	48	0	5	5	89.6%
1952 Rana clamitans	46	1	1	2	95.7%

C. ANALYSIS OF FALSE RESPONSES

1. FALSE POSITIVES

The ages of the women upon whom positive tests were falsely obtained ranged from 16 to 33 years with the exception of one woman who was 45 years of age. In only one case then do we find the possibility of a false positive due to high titers of FSH in a menopausal patient. Two other false responses were probably due to the re-use of previously

positive reactors, the positive test in each instance occurring only with the used animal. Two other cases may have possibly aborted without the knowledge of their attending physician. The reactions in each of these two cases was relatively strong. No explanation is suggested for the occurrence of false positives in the remaining 5 cases all of which were weak responses.

2. FALSE NEGATIVES

As indicated elsewhere 5 of the 8 false negative tests occurred during the summer of 1951 while the Rana pipiens frogs were being used. It is to the relative insensitivity of this animal that these false responses are attributed. No explanation is offered for the remaining 3 false negative tests.

D. PATHOLOGIC CONDITIONS

1. ABORTIONS

Referring to Table III it will be noted that 52 positive results subsequently resulted in abortions. Of these 49 were spontaneous abortions, 1 a therapeutic and 2 were criminal abortions. Of the spontaneous abortions 6 tests were characterized by weak responses. In 3 of these 6 the abortion occurred within a period of 4 days following the performance of the test. The exact date of abortion was not determined in the other 3 tests. 7 abortions occurred within a few days of the time of performing negative tests.

2. ECTOPIC PREGNANCY

4 cases of ectopic pregnancy were encountered in this series. 3 of these cases presented a positive response and the fourth a negative response. The experiences of several authors with the frog test and

ectopic pregnancy are summarized in Table VI where it will be noted that in approximately 50% of the cases false negatives will be encountered.

Table VI. The Frog Test in Ectopic Pregnancy

Author	Number of Ectopics	Number of Negatives	Percent Negative
Bromberg (31)	?	?	88%
Haskins (29)	14	7	50%
Holyoke (13)	7	3	43%
This Paper	4	1	25%
Average percentage of negative response			51%

3. OTHER CONDITIONS

One case of chorionepithelioma and two cases of hydatidiform mole are included in this series. The first is a case of a patient with metastases to the left lung. A complete hysterectomy and ovariectomy failed to reveal the primary source of the chorionepithelioma. A frog test run following the hysterectomy was positive with 3 cc of unaltered urine. No attempt was made to do a quantitative study at this time. A left pneumonectomy was performed. Following this surgery the frog test with unaltered urine was negative in each of two frogs. A Friedman run simultaneously was strongly positive in 1-40 dilution. The patient recovered. In each of the cases of hydatidiform mole strongly positive results were obtained with 1-1000 dilution of 3 cc of urine. In the first instance a subsequent examination 6 months after removal of the mole provided a negative

result. In the second instance increasingly weak responses were obtained for a period of 5 months after which the test became negative and has remained so through three subsequent bi-monthly examinations.

Our technique for performing rough quantitative estimations calls for the use of at least 8 frogs. Two frogs each are injected with 3 ml. of unaltered urine. 3 ml. each of dilutions 1/10, 1/100 and 1/1000 are injected into each of two frogs. A positive response with the 1/1000 dilution (0.003 ml. urine) coupled with suggestive chinical evidence from history and physical examination is considered diagnostic of either a mole or chorionepithelioma. A positive with the 1/100 dilution is considered suspicious; however, this response may occur during the first trimester of a normal pregnancy.

SUMMARY

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1. A partial review of the literature concerning the frog test for pregnancy is presented with particular reference to a.) the method of performing the frog test, b.) the mechanism of production of the gametokinetic response, c.) the expected levels of chorionic gonadotropin in normal and abnormal pregnancy conditions, and d.) an analysis of false responses.

2. Areas for further investigation into the source of false positive reactions are suggested. The author's series of 387 tests is reviewed and an analysis of the errors is attempted.

CONCLUSIONS

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1. The frog test for pregnancy is a rapid and valid method of diagnosis providing the following factors are considered:

   a. Technique including concentration of urine specimen or use of blood serum.

   b. Selection of the proper test animal with reference to seasonal variation and re-use of the animal.

   c. Patient's history.

2. The gametokinetic response may be produced by menopausal urine as well as by pregnancy urine. Whether or not this factor is eliminated by the use of the kaolin extraction method has not yet been definitely established.

3. A factor present in some jaundiced patients, possibly due to failure of the liver detoxification mechanism, produces false positive reactions.

4. Ovarian pathology may cause the production of false positive reactions.

5. It seems possible that the presence of pheochromocytoma might occasion false positive reactions. The frog test is suggested as a possible aid to diagnosis of this condition.

6. The normal pregnancy levels of chorionic gonadotropin are apparently sufficient at any stage after the 30th cycle day to

produce a positive frog test providing either the concentrated urine or serum technique is employed.

7. Approximately 50% of cases of ectopic pregnancy will be characterized by negative frog tests.

8. The diagnosis and prognosis of such pathologic states as abortion, chorionepithelioma and hydatidiform mole may be supplemented by the use of quantitative studies with the frog test.

APPENDIX

APPENDIX A

Scott Concentration Method (Modified)(34)

1. 100 ml. urine specimen in a 100 ml. graduated cylinder.
2. pH with p Hydrion paper.
3. Adjust to pH 4.0 with 20% HCl.
4. Add 5 ml. 20% kaolin suspension*. Shake well.
5. Allow to settle to 15 ml. and decant supernatant.
6. Pour remainder into 15 ml. centrifuge tube and centrifuge for 3 minutes at moderately high speed.
7. At this point the hormone is adsorbed to the kaolin. Discard supernatant.
8. To the kaolin residue add 5 ml. N/10 NA O H*. Mix well.
9. Centrifuge 3 minutes at moderately high speed. The hormone is now in the supernatant liquid.
10. Pour supernatant into a small flask, add one drop of phenolphthalein indicator.
11. Add 2% HCl until the red color disappears.
12. Pour into syringe and inject one half into each of two frogs.

* For urine specimens less than 100 ml., these reagents are correspondingly reduced. (i.e., 1 ml. each of 20% kaolin suspension and of N/10 NA O H is used for each 20 ml. of urine)

APPENDIX B

Cold Acetone Extraction (16)

1. Add 30 ml. urine to 100 ml. C.P. acetone.
2. Shake tube 3 minutes.
3. Centrifuge 4 minutes.
4. Discard supernatant.
5. Drain with tube inverted for 5-10 minutes.
6. Add 5 ml. M/150 Sorenson phosphate buffer, pH 7.5.
7. Inject one half into each of two frogs.

APPENDIX C

Ion Exchange Detoxification (37)

1. Preparation of resin for use.

   a. Amberlite XE-96 washed successively with 1% HCl, distilled water, 1% sodium carbonate and again with distilled water.

   b. Air dried and stored for use.

2. Detoxification of urine.

   a. 3 gm of resin added to 10 ml. urine in test tube.

   b. Shake vigorously and allow to settle.

   c. Supernatant used as injectate.

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