

Diagnosis of pregnancy in women attending the General Hospital, Ipoh, Perak

by *J. T. Ponnampalam*

MD (Belfast), M. Sc. (S'pore)

Senior Pathologist,
Institute for Medical Research,
Ipoh,
Perak.

Introduction

IN RECENT YEARS, there has been a widespread trend away from the biological tests for pregnancy. In its place, various slide tests have been introduced which are claimed to be more accurate and rapid than the traditional animal tests, e.g. latex agglutination-inhibition test, complement fixation and a precipitin technique.

In the tropics, kits for immunologic methods deteriorate more rapidly if not maintained under suitable conditions of refrigeration. This, in turn, affects the tests giving rise to false positives. Besides, the cost of immunologic tests is greater than the cost of biologic methods using the male toad in preference to other laboratory animals.

This paper reviews the results obtained by the examination of 85 specimens of urine from 73 patients with normal or disturbed pregnancies using the toad test, gravindex and prepuerin. The purpose of this investigation is to determine which of these tests gives an accurate indication of the state of pregnancy and whether or not the foetus is viable as opposed to the mere presence of placental tissue. One thousand other cases were

examined using the toad test only and this test was found to provide reliable information of use to the clinicians except in under four per cent of cases where a confirmatory test was necessary using gravindex and/or prepuerin.

Review of Literature

Some three thousand years ago, the Egyptians attempted to diagnose pregnancy in the human by watering the seeds of wheat and other cereals with the urine of women. If these seeds germinated, the woman was considered to be pregnant. Hendriksen (1941) tried out this early Egyptian method and found that 75% were correctly positive and 85% correctly negative — a degree of accuracy which was no worse than many, and even better than some of the recent tests carried out within the last 40 years.

In 1928, Ascheim and Zondek demonstrated the presence of gonadotrophic substances in the urine of pregnant women and provided the basis for considering the placenta an endocrine organ. They called these substances prolan which was probably made up of several different fractions. It was not until 1938, however, that the hormone

DIAGNOSIS OF PREGNANCY IN WOMEN

(chorionic gonadotropin) was isolated from placental cells growing in tissue culture.

The early tests were mainly confined to the qualitative diagnosis of pregnancy but later, the quantitative estimation of the hormone was useful as an aid in the diagnosis of hydatidiform mole or choriocarcinoma.

The various tests used in the detection of pregnancy in recent years may be divided into (I) Biological tests, and (II) Immunological tests.

(I) Biological tests.

(a) **Asheim Zondek test:** Five female immature white mice, 18 to 20 days old and weighing 5 grams to 7 grams, were used for this test. 0.4 ml. of urine were injected subcutaneously into each animal three times daily for two days for a total dose of 2.4 ml. One hundred hours after the first injection, the animals were killed and the ovaries examined for corpora lutea.

This test confirms the presence of human chorionic gonadotrophin in the urine. It is produced by the placenta and should be distinguished from pituitary gonadotrophin; the latter could also produce effects on the ovaries similar to that produced by the former, thereby giving rise to false AZ reactions. A year later, after the publication of Asheim and Zondek's results, Professor F.A.E. Crew and Dr. B.P. Wiesner set up the first laboratory concerned with the diagnosis of pregnancy in Edinburgh. Over 800 Asheim Zondek tests were carried out in this laboratory with an accuracy, based upon the clinical diagnosis, of 97.8% (Crew 1930).

Dilution or Semi-Quantitative Tests: These tests were done if the pregnancy was thought to be abnormal. Asheim (1930) observed that women with hydatidiform mole excreted more chorionic gonadotrophin than women with normal pregnancies. Dilution tests provide additional information when attempting to differentiate between pregnancy, hydatidiform mole and chorionepithelioma. Dilution tests are most useful during the period of follow-up of these cases. Biological tests are less sensitive than immunological tests in detecting HCG activity in urine of cases of normal pregnancy and hydatidiform mole (Wide and Hobson, 1964).

(b) **Friedman test:** A mature female rabbit that has been isolated for 3-4 weeks is used. Ten ml. of urine are injected into the ear vein. At the end of 24 hours, the animal is anaesthetised and the ovaries examined for ruptured haemorrhagic follicles which indicates a positive reaction. Some of the animals show a positive result after 24 hours; if negative, the animals should be

examined again after a further 24 hours for delayed reactions.

The animal may be used again at the end of six weeks, or it may be killed after one test. The prolonged animal husbandry, or the cost of acquiring new animals for regular use in the laboratory, has resulted in the test being replaced by the more recent conventional methods. Friedman and Lapham (1931) reported a 100% accuracy for the test after examining 92 specimens of urine from pregnant and non-pregnant women.

(c) **Rat hyperaemia test:** Two immature female rats are used. 5 ml. of urine are injected subcutaneously into each animal at least four hours apart. The animals are killed the following day. A positive result is indicated by an enlarged and reddened ovary. A negative result is indicated by a small pink ovary. It is sometimes difficult to detect the degree of pink which is diagnostic of a positive test, and a high level of accuracy can only be maintained by skilled technicians experienced in reading the results. The end point of each test was read at 2, 6 and 24 hours after injection. The test had an accuracy of 87% at 2 hours and 99% at 24 hours (Zondek, Sulman and Black 1945). Berman (1956) has reviewed the literature on the many modifications of this technique.

(d) **Hogben test. (Female South African Clawed Toad, *Xenopus Laevis*):** Hogben (1930) showed that the injection of extracts of the anterior lobe of the ox pituitary (ALP) stimulated ovulation and oviposition in *Xenopus laevis*. These findings were later confirmed by Bellerby (1933), Shapiro and Zwarenstein (1934). At the Pregnancy Diagnosis Laboratory, Edinburgh, 350,000 Hogben tests were done between 1948 to 1964, based upon the subsequent clinical diagnosis in 71,000 tests. It was shown that this test had an accuracy of 99.3% (Hobson, 1966). A number of workers have not been able to maintain the toad in good condition and are, therefore unable to get consistent results. Also, as the female toad is less sensitive than other animals used in biological tests, the urine has to be concentrated by the method of Scott (1940) and this could be time consuming. If laboratory conditions are not standardised, the *Xenopus* will lay eggs spontaneously. An occasional false positive result is also obtained in women receiving chlorpromazine therapy as shown by Marks and Shackcloth (1963).

(e) **Galli-Mainini test. (Male frog or toad test):** Galli-Mainini (1947) showed expulsion of spermatozoa by this animal after an injection of urine from a pregnant woman. The test had an accuracy

of 94% to 95% and both false positive and negative reactions occurred.

II. Immunological tests.

Agglutination inhibition tests: Urine containing human chorionic gonadotrophin will inhibit agglutination of HCG sensitised red cells or latex particles by rabbit — anti HCG sera. Some urines contain substances which prevent the inhibition of agglutination by HCG. For this reason, a control test is always done. The control consists of formalin preserved erythrocytes, stabilised in normal rabbit serum but not sensitised with HCG. Agglutination of this control should always be inhibited by urines from pregnant and non-pregnant women. If agglutination still occurs, then non-specific agglutinins must be present and these can be eliminated by acetone precipitation of the urine. The precipitate is washed with ether and alcohol, dried and resuspended in a phosphate buffer solution. This solution is centrifuged, and the supernatant liquid is tested again.

Commercially prepared haemagglutination inhibition tests.

(a) **Pregnosticon** (Organon Laboratories Ltd): This test detects a concentration of 1000 i.u. HCG per ml. of urine, and is read by observing the pattern of sedimented cells. The accuracy of the test varies from 98.7% to 99.8%. False negative tests are due to:

- (i) Low levels of HCG in the urine which is below the sensitivity of the test.
- (ii) Certain unidentified substances in the urine which interfere with the test.
- (iii) Non-specific antibodies in the urine.
- (iv) Glassware contaminated with soap or other detergents.

(b) **Prepuerin.** (Burroughs Wellcome and Co.): The urine is filtered and dilutions of 1:5, 1:10 and 1:20 prepared with an isotonic borate buffer. A positive control and a negative control were set up with each test. After mixing, the test is left overnight before positive reactions can be read with certainty. A reaction is recorded as negative if agglutination has occurred in all the tubes containing urine and the test suspension. A positive reaction is one where there is no evidence of agglutination but the cells have collected at the bottom of the tube in the form of a button with a clear centre. The overall sensitivity of the test is not as high as that of pregnosticon. Using the prepuerin test a positive reading in a dilution of 1:5 indicates a urinary concentration of 1,000-2,500 i.u. per litre.

(c) **Urinary chorionic gonadotrophin test:** (Denver laboratories). This test is not as accurate as the above two and gives many false negative reactions.

(d) **Ortho Gravindex — Slide test:** Latex particles coated with HCG are used instead of sheep erythrocytes. One drop of antiserum is added to a black slide and this is mixed with one drop of urine to be tested using an applicator stick and mixing for 30 secs. A drop of the latex particle suspension is then added and the slide gently rotated for two minutes at which time the reading is taken. If agglutination occurs, there is no evidence of pregnancy. If there is no agglutination, the test is positive, i.e. the woman is pregnant.

This is a rapid test but many false negatives occur. If the test is positive i.e. no agglutination occurs, it is more difficult to read if the light is poor, and the latex particles themselves give a false impression of agglutination. If agglutination occurs, the end point is easy to read. The test is able to detect a concentration of 5000 i.u./litre of HCG in the urine. The best results are obtained between 41 and 109 days after the last menstrual period. Sato & Greenblatt (1965) found that 60% of false negative reaction occurred in early pregnancy, but there were no false positives.

Materials and Methods.

The tests routinely carried out at the pregnancy testing laboratory, Institute for Medical Research, Ipoh are the male toad test, Gravindex test (Ortho) and the Prepuerin (Burroughs Wellcome).

(1) **The male toad test:** The toad commonly found in Malaya and other countries of South-east Asia is *Bufo Melanosticus* which is readily available all the year round in Malaya. It is found in large numbers along rivers and adjacent swampy areas, particularly after a heavy rainfall. The male is identified by the yellowish-orange patch under the chin, and the inside fingers of the forelimbs are black on top. It also croaks fairly readily. They are easy to catch at night with the aid of a torch when they come out to feed on insects near street lamps.

Animal Husbandry.

Housing: They were kept in the rear yard in a brick enclosure 3 feet x 4 feet x 2 feet, the top being partly covered with planks to keep out the sun and partly with wire gauze to permit adequate ventilation and lighting, and at the same time to prevent their escape. There was water to a depth of 4 inches with a few stones to serve as islands on which the toads could rest. All

DIAGNOSIS OF PREGNANCY IN WOMEN

toads used for the tests (whether these were positive or negative) were released in the gardens surrounding the laboratory which is at a considerable distance from the collecting area. Animals were never kept in the tank for longer than 10 days as they were found to lose weight in captivity in spite of the diet being adequate. The tank is thoroughly cleaned once or twice a week.

Feeding: The animals were fed weekly or twice a week. The food consisted of earthworms, maggots, small quantities of boiled rice, and young sprouted beans. The maggots obtained from fish entrails were kept on the rest platforms in the tank until flies were attracted to lay their eggs.

Economy: The toad was the most economical animal available for use in the biological test as it was very easy to collect and the cost of feeding was very low. The collector was paid 30 cents (Malayan = 4d. sterling) for each animal, and at a cost of 30 cents, about 20 animals could be fed each week. Three toads were used in case some of the animals were insensitive. The cost of each test was approximately 90 cents (Malayan) — (14p sterling). About 10 tests were carried out each week.

Definite end point: The overall accuracy of the pregnancy test depends upon the clarity of the end point. With the toad test, the production of spermatozoa gives a definite end point easily recognisable and which does not require a high degree of skill in identification or carrying out the test.

Rapidity of Reaction: Most urines gave a positive reaction within $\frac{1}{2}$ hour — 2 hours of injection. Toads, which were negative, were examined again at the end of 4 hours.

Urine: For the qualitative diagnosis of pregnancy, it had been the practice for several years to collect the untimed first morning specimen. Recently random specimens were accepted which were more convenient to the patient if she had not been instructed to collect the morning specimen, and the results were known sooner. Many of these patients came from considerable distances away and it was not always practical for them to bring their urine specimens with them. It was more convenient to collect a random sample on their arrival at the laboratory. No significant differences were found in the tests whether random or first morning specimens were used, provided fluid intake was reduced to a minimum. Further, there was less contamination and false results were avoid-

ed if the tests were done immediately after collection. No concentration techniques were used for the following reasons:

1. It was time consuming and there was a shortage of trained personnel.
2. In a preliminary survey carried out in this laboratory, it was found that similar results were obtained after concentration of the urine as after the injection of 5 ccs. of neat urine diluted with an equal volume of sterile physiological saline.

Some of the urine specimens killed the toad and the following procedure was adopted to minimise this:-

- (a) The containers used for the collection of the specimens were thoroughly cleaned by boiling in plain water for 10 minutes and then rinsed in tap water, followed by distilled water. No soap or detergent should be used for the cleaning.
- (b) All urines were refrigerated from $\frac{1}{2}$ - 1 hour to allow precipitation of the phosphates and any other toxic substances which may be present.
- (c) The urine was then filtered into a wide mouth test tube of 50 cc. capacity. All glassware were cleansed using the procedure mentioned above.
- (d) Heavily contaminated specimens were rejected as these caused disturbances in the reading of the gravindex and prepuerin tests and were rather toxic to the toad.
- (e) After filtration, an equal volume of physiological saline at room temperature was added and 10 ccs. of this diluted urine was injected intraperitoneally into the toad.

During the test, the animals were kept in separate containers. Three animals were used for each test. The containers were in the form of glass tanks measuring 10 in x 7 in x 6 $\frac{1}{2}$ in. A drop of cloacal fluid was removed from each of the animals with a fine tip pasteur pipette, which was inserted into the cloaca and gently moved up and down. Sufficient amounts of cloacal fluid were produced within 60-90 secs. This was placed on glass slides and examined under the microscope for spermatozoa.

Site of Inoculation.

- (1) **Dorsal lymph sac.** — Absorption was

slower and it was not possible to inject more than 3-5 ccs. of urine by this route. Also, the animal succumbed more readily to toxic substances in the urine if this route was used.

(2) **Hind limb.** — The skin is loose over this area and some of the fluid injected escaped through the perforation made by the needle. It was not possible to inject more than 5 ccs. by this route.

(3) **Intraperitoneal route.** — We have found this very satisfactory for the following reasons:

- (a) The toad can easily tolerate the volume of 10 ccs. that was injected. After injection, the site was gently squeezed between the thumb and index finger thereby sealing the aperture made by the needle and preventing the escape of the injected fluid.
- (b) A repeat 5 ccs. can be given at the end of 4 hours if the reaction was negative and the clinical history and examination were suggestive of pregnancy.
- (c) The action on the testes was more direct and spermatozoa were produced in a shorter time than by any other route.

After injection, the animals were kept in dry tanks without any water so that no exchange of fluids will take place between the external and internal environment thereby preventing any loss in chorionic gonadotrophin. After the test was over, the animals were released far from the site of collection.

Gravindex Slide test (Ortho): Refrigerated and filtered urine was used for this test. All reagents and urine stored in the refrigerator were kept at room temperature for $\frac{1}{2}$ hour before proceeding with the test, details of which have been described above.

There was a high incidence of false positive results with this test in this country. The main reason for this had been the inadequate refrigeration facilities, resulting in deterioration of the reagents, particularly when the test kits were ordered through the local dealers and supplies were stored under unsatisfactory conditions. When supplies were forwarded direct to the laboratory by the manufacturers under refrigeration, the results were found to be very satisfactory and false positives were reduced to a minimum. If the test was positive, no agglutination occurred, while negative reaction was associated with agglutination.

One disadvantage of this test was that traces of agglutination were sometimes difficult to detect. Considerable experience was required in reading the end points and deciding which test was positive and which was negative. The urine should be as fresh as possible with minimum bacterial contami-

nation. All glassware should be scrupulously clean, and only those specially cleaned by the laboratory were used.

Prepuerin (Burroughs Wellcome): Fresh urine was used in the test after refrigeration and filtration and dilutions of 1:5, 1:10 and 1:20 were prepared with an isotonic borate buffer as described elsewhere in this paper. A positive reaction could be read in 4 hours but it was preferable to read the test overnight when there was no evidence of agglutination and the cells collected as a button at the bottom of the tube. The rack holding the test tubes should be subjected to as little disturbance as possible in order to reduce the incidence of false reactions to a minimum.

Results

Eighty-five specimens of urine were examined from 73 women of the three major races, i.e. Indians, Chinese, and Malays and they were followed up till the end of their pregnancies. Their clinical histories were variable and tests for the presence of human chorionic gonadotrophin were carried out for the following clinical conditions:—

- (1) Diagnosis of pregnancy.
- (2) Molar pregnancy.
- (3) Threatened abortion.

In the past, diagnosis of pregnancy was done more by clinical examination than by laboratory methods. At present, both the medical profession and the laity wish to have results available immediately for various reasons. When the woman is receiving treatment for infertility, the first missed period would lead her to visit the obstetrician who may require a pregnancy test to confirm or disprove any findings suggestive of pregnancy.

In these cases studied, it was found necessary to examine more than one specimen of urine from a few of them. Many of the patients gave irregular histories of amenorrhoea and/or bleeding per vagina so that it was difficult to know the exact date of the last missed period. One of the possible causes of this irregularity in the menstrual cycle was probably the result of the indiscriminate and unsupervised ingestion of drugs used for birth control purposes.

As many of the cases were from rural areas, with inadequate means of communication, it was not possible to follow up more cases up to the time of termination of the pregnancies. In 64 cases, the three pregnancy tests gave similar results which confirmed that the patients were pregnant. However, in nine cases, the pregnancy tests gave contradictory results. The prepuerin test was found to be the most sensitive and also gave false positive

DIAGNOSIS OF PREGNANCY IN WOMEN

results. In cases of threatened abortion, the first indication that intra-uterine death had occurred was a negative toad test, while the gravindex and prepuerin tests continued to be positive. In two other cases, the toad test gave false negative results while the other two were positive and both these delivered normal children. In some cases of abortion, the prepuerin test showed a disagglutinated pattern with crenated edge and it was not possible to decide whether the test was positive or negative in experienced hands. In one case of inevitable abortion, the gravindex and prepuerin tests were positive three days after curettage while the toad test was negative.

In a separate series of one thousand cases, where the toad test only was used, the obstetrician reported an overall accuracy of 96%.

Although the number of cases studied were few, the results seem to suggest that no one test is adequate in the diagnosis of pregnancy, and that more than one test should be used. The claims made for the superiority of the prepuerin and gravindex tests over the toad test does not appear to be justified in terms of cost, interpretation of the results, and rapidity with which the tests may be performed.

Interpretation of results is an important feature in the use of pregnancy tests and the simplest to interpret is the toad test where a positive or negative case shows the presence or absence of spermatozoa respectively. In terms of cost, the toad test is the cheapest as the animals are available in any part of the country. The annual cost of gravindex and prepuerin approximates to about 30 per cent of the total annual recurrent expenditure

References

- Aschheim, S. & Zondek, B. (1928) Schwangerschaftsdiagnose aus dem Harn. (Durch Hormonnachweis). *Klin. Wschr.* 7, 8.
as quoted by B.M. Hobson, J. *Reprod. Fert.* (1966) 12, 33-48.
- Aschheim, S. (1930). The early diagnosis of pregnancy, chorionepithelioma and hydatidiform mole by the Aschheim-Zondek test. *Am. J. Obstet. Gynec.* 19, 335.
as quoted by B.M. Hobson, J. *Reprod. Fert.* (1966) 12, 33-48.
- Bellerby, C.W. (1933). The endocrine factors concerned in the control of the ovarian cycle. I. *Xenopus laevis* as a test animal. *Bioche. J.* 27, 615.
- Berman, R.L. (1956). A critical evaluation of biological pregnancy tests. *Am. J. Obstet. Gynec.* 72, 349.
- Crew, F.A.E. (1930). Pregnancy Diagnosis Station. Report on first year's working. *Br. Med. J.* 1, 993.
- Friedman, M.H. & Lapham, M.E. (1931). A simple, rapid procedure for the laboratory diagnosis of early pregnancies. *Am. J. Obstet. Gynec.* 21, 405.
- Galli-Mainini, C. (1947). Pregnancy test using the male

of a clinical pathology department, if these tests are used routinely.

In many hospitals in the country, hospital assistants, with very little laboratory training, are required to be in charge of laboratories and the toad test is easy to perform under these circumstances.

In difficult cases, more than one pregnancy test may be used, otherwise the use of the toad test should provide reliable information in the vast majority of cases. In some cases of false negatives, the toad test should be repeated.

In cases of molar pregnancy, a positive toad test of 1:32 dilution of urine and above was considered diagnostic; with gravindex, the titre was 1:64 and above. According to the manufacturers, with the prepuerin test, a dilution greater than 1:400 was regarded with suspicion. The above titre should be persistently elevated after the 5th day of delivery before a presumptive diagnosis of molar pregnancy can be made.

Conclusion.

A preliminary survey was carried out, using three pregnancy tests, viz toad, gravindex and prepuerin. Under local conditions, the toad test did not appear to be significantly inferior to the other two. But in terms of cost, and availability of trained laboratory personnel to interpret these tests correctly, the toad test was preferable on account of the simplicity in performing and reading the test, and the decreased cost. Although false negatives occurred with the toad test, it never gave a false positive in our experience.

- toad. *J. clin. Endocr. Metab.* 7, 653.
- Henriksen, E. (1941). Pregnancy tests of the past and present. *West. J. Surg. Obstet. Gynec.* 49, 567.
- Hobson, B.M. (1966). Pregnancy Diagnosis. *J. Reprod. Fert.* (1966) 12, 33-48.
- Hogben, L. (1930). Some remarks on the relation of the pituitary gland to ovulation and skin secretion in *Xenopus laevis*. *Proc. R. Soc. Afr.* 5, 19.
- Marks, V. & Shackcloth, P. (1963). Tranquilliser and pregnancy tests. *Br. med. J.* 1, 52.
- Sato, T. & Greenblatt, R.B. (1965). Detection of early pregnancy. Immunologic test versus the rat hyperemia test. *Am. J. Obstet. Gynec.* 91, 31.
- Scott, L.D. (1940). The concentration and detoxification of human urine for biological pregnancy diagnosis. *Br. J. expt. Path.* 21, 320.
- Shapiro, H.A. & Zwarenstein, H. (1934). A rapid test for pregnancy on *Xenopus laevis*. *Nature, Lond.* 133, 762.
- Sulman, F. & Black, R. (1945). The hyperemia effect of gonadotrophins on the ovary, and its use in a rapid pregnancy test. *J. Am. med. Ass.* 128, 939.