PREGNANCY DIAGNOSIS — HAEMAGGLUTINATION INHIBITION METHOD (PREPUERIN) COMPARED WITH THE *XENOPUS LAEVIS* TEST

W. M. POLITZER, M.D., *South African Institute for Medical Research, Johannesburg*

The human placenta secretes a gonadotrophic hormone which has luteotrophic and luteinizing activity.[1] Increased excretion of human chorionic gonadotrophin (HCG) in the urine forms the basis of all recognized laboratory pregnancy tests.

The *Xenopus laevis* pregnancy test[10] has been carried out by this Institute for 14 years. The frog ovulates when injected with a urine extract containing 3,000 IU/l. as a minimum concentration,[2] which, according to some authors,[3] may be reached 24 - 30 days after conception. Edward[4] found that at about 6 weeks after the last menstrual period the urinary level of HCG may be 5,000 IU/24 hours. Peak excretion (up to 500,000 IU/24 hours) occurs during 7 - 13 weeks after the last menstrual period and seldom lasts longer than 10 days. For the remainder of pregnancy the level ranges between 4,000 and 11,000 IU/24 hours. In late pregnancy values of 2,000 IU/24 hours may be encountered, and in such cases the *Xenopus laevis* test becomes negative. Following delivery, human chorionic gonadotrophin normally disappears and is absent from the urine after 1 week.[1] If intra-uterine death has occurred the test may give a false positive result for a few days or even weeks.[1] In ectopic pregnancy fewer eggs are extruded.[5]

A hydatidiform mole produces vast amounts of HCG, which are excreted for longer than the 10-day peak,[4] and when choriocarcinoma exists after the expulsion of a mole or termination of a normal pregnancy, persistent high values are encountered.[1] If either of these conditions is suspected the concentrated urine is subjected to the same treatment as that for a pregnancy test, while 1:10 and 1:20 dilutions are injected directly into the frogs. Positive results in the dilutions usually point to the presence of a growth.

It is important to mention the accuracy of the *Xenopus laevis* test; for example Weisman and Coates[5] over 5 years investigated more than 1,000 cases, giving the following results:

Correct positive results	673
Correct negative results	316
Incorrect positive results	0
Incorrect negative results	11

At the time of testing the 11 subjects who gave incorrect negative results were less than 14 days overdue in their menses. When reinvestigated 7 - 10 days later all 11 gave positive results.

The disadvantages are that the frogs have to be accommodated in a thermostatically controlled room with a sufficient number of tanks to avoid overcrowding. After injections the frogs should rest for at least 4 weeks before being re-used, thus necessitating a large colony of frogs as well as staff to maintain them. The life-span of the female frog used for this purpose is 5 - 6 years and the mortality rate is increased by 'red legs' (a septicaemic disease caused by *Bacillus hydrophilum-fuscus*) and occasionally a fungus infection.

In this laboratory it has been found that positive results may be obtained approximately 14 days after the first missed period. On rare occasions it has been necessary to resort to the Aschheim-Zondek[6] test, which may give a positive result at a slightly earlier stage of pregnancy. When using the rat test, observing hyperaemia of the ovaries, pregnancy could be detected 24 days after the last menstrual period.[3]

While the adopted methods for the detection of HCG in urine for the last half century were based on biological procedures, inevitably new methods would be introduced representing a complete departure replacing the use of laboratory animals.

With the recent introduction of immunological tests for pregnancy, it was felt desirable to conduct comparative stu-

dies to determine the accuracy of the new methods placed at our disposal. For the purpose of this study the one-stage haemagglutination inhibition (HI) test was selected. The principal reaction of the HI test is also the detection of HCG in pregnancy urine, determined by means of an antigen-antibody reaction. In the HI method the antigen HCG is adsorbed on to the surface of sheep erythrocytes. These treated cells will then behave as the specific antigen and are therefore agglutinated by the corresponding antibody. The agglutination pattern appears as a uniform, opaque, diffuse sediment at the bottom of the glass tube. If the antigen is present in the urine this will combine with the antibody and inhibit agglutination. The red cells then appear as a reddish-brown ring in the centre of the bottom of the tube.

MATERIALS AND METHODS

One hundred and seven specimens of urine submitted to this Institute for the pregnancy test were used in this trial. On each specimen the *Xenopus laevis* test and the HI test ('prepuerin') were carried out in parallel.

1. The precipitation method used for the *Xenopus laevis* test was that described by Scott.[7] Each of 4 frogs were injected with 2·5 ml. of extract corresponding to 50 ml. of urine instead of 25 ml. of urine, thereby increasing the sensitivity of the test. Ova extrusion occurred in 4 - 17 hours; however, observation continued for a total of 48 hours to ensure that delayed ova extrusion was not missed.

2. The HI test system consisted of a test and control suspension:

(*a*) Test suspension: Formalin-preserved sheep erythrocytes sensitized with HCG. When agglutinated by rabbit anti-HCG a completely agglutinated pattern develops when the suspension is added to a simple diluent.

(*b*) Control suspension: Formalin-preserved sheep erythrocytes stabilized in normal rabbit serum but not sensitized with HCG.

The system must not be used to identify HCG in serum or body fluids other than urine.

Both suspensions are supplied in a freeze-dried form in 3·5 ml. bottles and are stable for at least 12 months in this state at 2 - 10°C. For use it is essential to reconstitute in isotonic buffer by the addition of 3·5 ml. of borate buffer saline and not in distilled water. The reconstituted material is stable for one week at 0 - 4°C.

A buffer pH 8·2 - 8·3 is necessary and comprises 3·0 G. of crystalline borax ($Na_2B_4O_7.10H_2O$); 4·4 G. of boric acid (H_3BO_3) and 7·6 G. of sodium chloride (NaCl) made up in 1 l. of distilled water. The three compounds are available in tablet form and are made up to volume using 1 tablet for each 20 ml. required, the tablet being dissolved in distilled water.

The method was as follows: Early-morning specimens of urne were centrifuged and from the supernatant 1:5, 1:10 and 1:20 dilutions were made with the buffer.

1: 5 = 0·5 ml. supernatant+2 ml. buffer.
1:10 = 0·5 ml. supernatant+4·5 ml. buffer.
1:20 = 0·5 ml. supernatant+9·5 ml. buffer.

Of each dilution, 1 ml. was pipetted into 3 different tubes, preparing simultaneously a parallel series. The tubes were 3×0·5 inches with round bottom. It is important that there should be no flaws since they may interfere with the formation of the pattern. To all tubes in the first series 0·1 ml. of test suspension was added and to the second series 0·1 ml. of control suspension was added.

In addition, with each group of tests, two tubes each containing 1 ml. of buffer only were included as reagent controls to which 0·1 ml. of test and control suspension were added respectively. After gentle shaking the tubes were set up in a test tube rack.

For the purpose of this trial a special rack was designed, fitted with an adjustable mirror to facilitate reading the results without disturbing the precipitate formed (Fig. 1).

The tubes were left standing at room temperature (in cold, unheated rooms false agglutination may occur owing to cold agglutinins in the urine), undisturbed by draughts and vibration and not exposed to sunlight. A convenient time for setting up of the test was found to be 2 p.m. so that results could be read early next morning. It was found that positive results in some cases could be read after 4 hours; but before deciding on a negative result, it was felt advisable to wait until the following morning.

All glass-ware used for this test had to be prepared in accordance with accepted principles as used in any haematological studies. Glass tubes should be cleaned with a brush and distilled water. No detergents or cleaning agents should be used other than 2% sodium hydroxide followed by 2% hydrochloric acid and thorough washing in distilled water.

Reading of Tests

Interpretation of the agglutination patterns is illustrated as follows:

(*a*) *Non-pregnant.* Complete agglutination in all dilutions of test suspension with inhibition of agglutination in all controls.

(*b*) *Pregnant.* If the test suspension is treated with urine containing HCG in sufficient concentration, agglutination is inhibited and a pattern develops comparable with that of the unsensitized control suspension. Inhibition of agglutination in test suspensions may occur in all 3 dilutions. This is the most frequently encountered pattern. Inhibition at 1:10 and/or 1:5 dilutions would indicate reduced HCG levels. The result would be recorded accordingly. Inhibition of agglutination will always occur in all controls.

(*c*) *Pregnant.* Occasionally complete agglutination occurs in test and control in the 1:5 dilution with inhibition of the 1:10 and 1:20 dilutions of test and control suspensions indicating pregnancy with non-specific agglutination at the 1:5 level (Fig. 2).

RESULTS

The results are shown in Table I.

In the 14 cases where discrepant results were obtained follow-up studies were conducted in order to determine the accuracy of the two tests. Of 13 giving negative *Xenopus laevis* test results one patient died of malignant hypertension and uraemia 2 weeks after the specimen of urine was collected. Examination per vaginam excluded pregnancy (a postmortem examination was not performed).

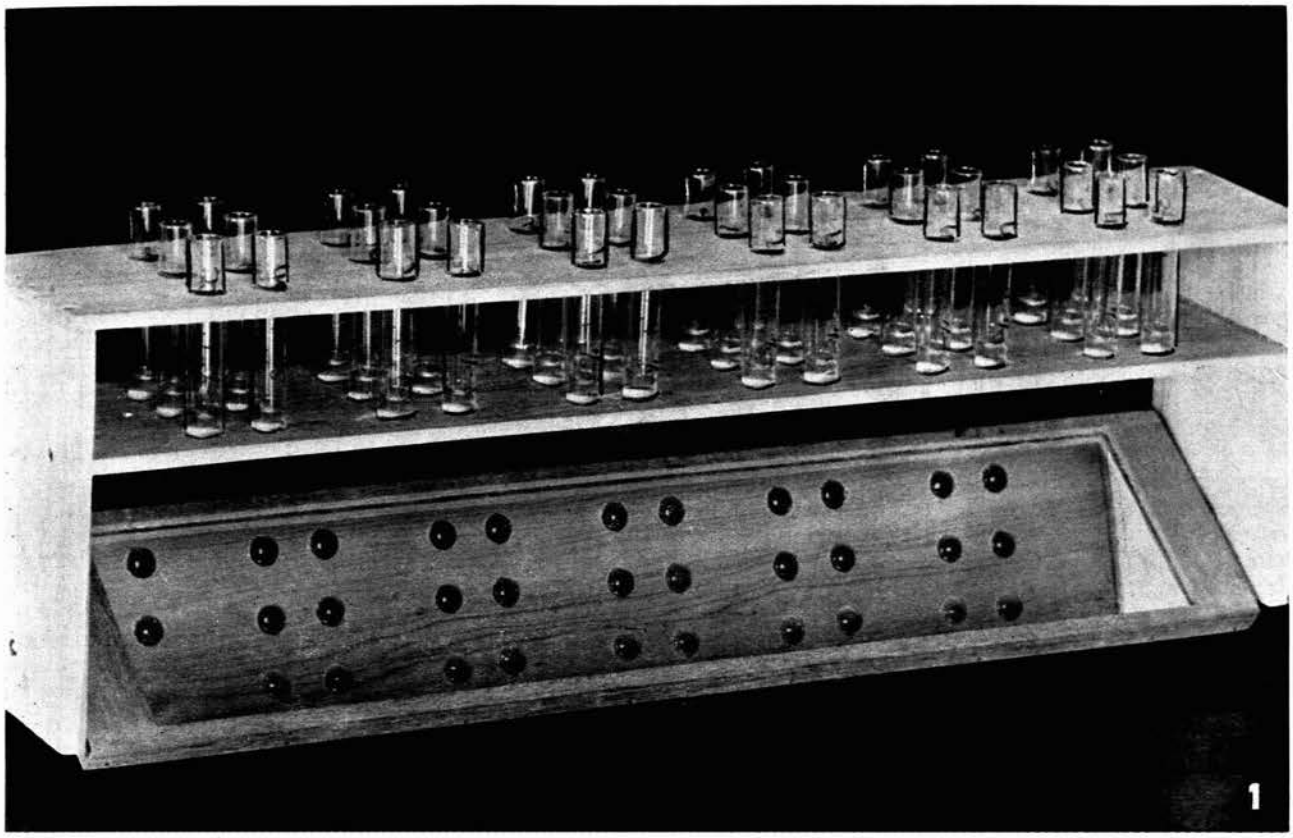

Fig. 1. Special rack for test and control suspensions.

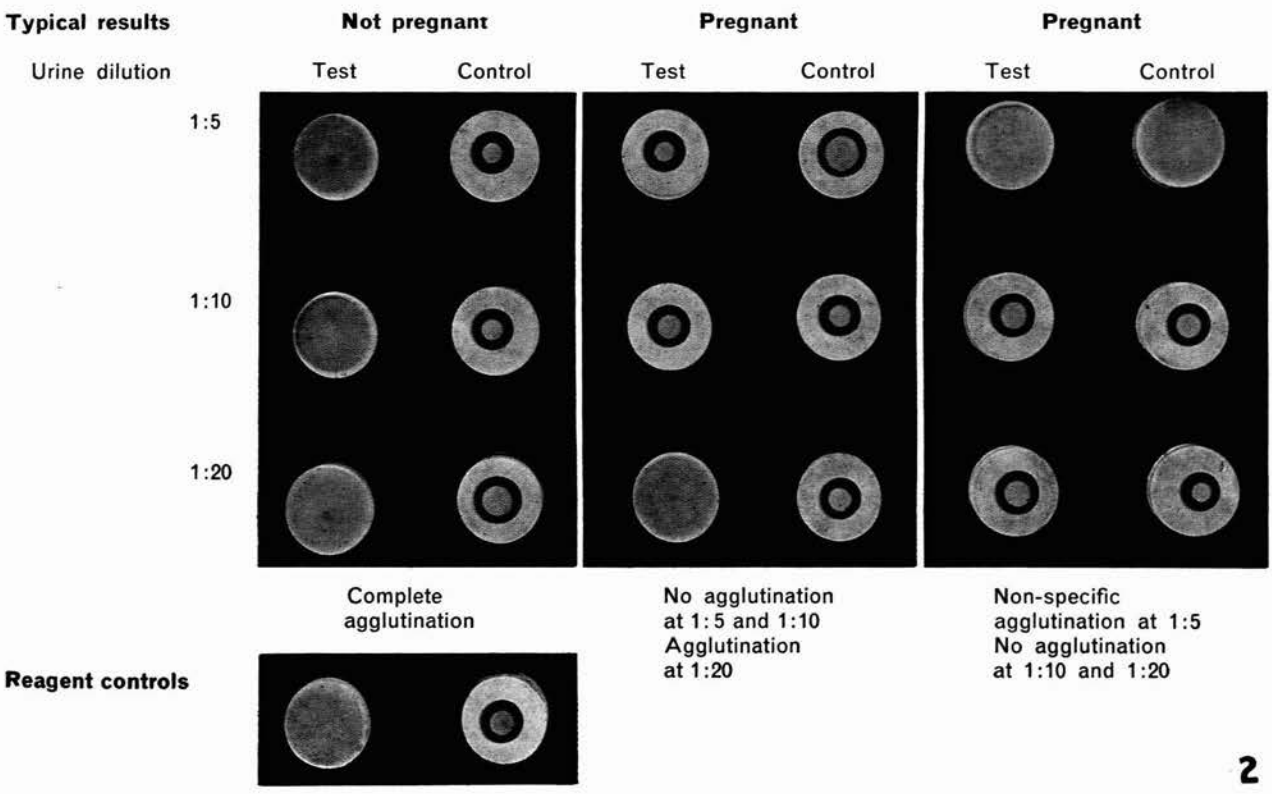

Fig. 2. Typical haemagglutination patterns.

The four-colour illustrations on the reverse side of this page were sponsored by Burroughs Wellcome & Co. (S.A.) Ltd.

TABLE I. COMPARISON OF RESULTS OF *XENOPUS LAEVIS* TEST WITH HAEMAGGLUTINATION INHIBITION TEST

Number of specimens	Xenopus laevis *test results*		*Haemagglutination inhibition test results*	
	Positive	*Negative*	*Positive*	*Negative*
40	40	—	40	—
53	—	53	—	53
13	—	13	13	—
1	1	—	—	1

Seven cases were subsequently found to be normal pregnancies. The remaining 5 justify special mention and the results obtained in 3 dilutions are shown in Table II.

TABLE II. VALUE OF HI TEST IN DIAGNOSIS

Patient	*HI result Dilutions*			*Patient's condition*
	1/5	1/10	1/20	
1	+	+	—	Presented as ? appendicitis. Laparotomy showed ectopic pregnancy
2	+	—	—	Urine collected in the morning. Patient aborted same afternoon
3	+	—	—	Amenorrhoea for 10 days. Patient aborted 2 days after urine was collected
4	+	+	+	Amenorrhoea 7 weeks. Abdominal pain when urine submitted. Aborted 3 weeks later
5	+	+	+	Bleeding p.v. and shedding cysts for 3 weeks when urine was collected

The final discrepant result giving a positive *Xenopus laevis* and negative HI result was found to have been 14 weeks pregnant when the urine was collected. Although apparently contradictory results were obtained in the cases listed in Table II, a close study will show that in actual fact the results of both tests were correct. The *Xenopus laevis* test is less sensitive and will therefore give a negative finding earlier than the HI test in cases of abortion and a disintegrating mole. In this laboratory it has been found that in ectopic pregnancy the *Xenopus laevis* test often remains negative.

Thus the accuracy obtained was as follows:

	Xenopus laevis *test*	*HI test*
Correct positive	85%	98%
Correct negative	100%	98%

DISCUSSION

In 1963 Fulthorpe *et al.*[8] conducted a comparison study utilizing the male toad *Bufo bufo* and the HI test. Three hundred and eighty-eight samples were tested of which 350 results were in complete agreement. A final analysis of the results in that series showed 98·2% accuracy with the HI test against 92·8% obtained with the male toad test. It is important to observe that in their investigation non-pregnant controls totalling 211 were included, and of this number 54 were of the age group 50 - 59 years whose pituitary gonadotrophin excretion is increased. It would appear, therefore, that the HI test is specific for HCG.

The criteria for the acceptance of an alternative method for the detection of HCG must be a test at least as accurate and sensitive as the *Xenopus laevis* test and it should be of value in cases of hydatidiform mole, choriocarcinoma, some malignant tumours of the testis, as well as in pregnancy. Progressively diminishing excretion rates of HCG have been reported in cases of ectopic pregnancy.[9] Three dilutions — as done in this series — may assist in making a diagnosis (Table II).

An extremely important observation made was the ability of the HI test to determine early pregnancy at a time at which the HCG level in the urine was less than 3,000 IU/l. Three cases fell into this category, giving in each case a positive result at a titre of 1:5 only.

As already mentioned, peak HCG levels are reached at 7 - 13 weeks, and levels as high as 500,000 IU/24 hours have been recorded. These levels dwindle to the point where again less than 3,000 IU/l. may be excreted. Five cases in this study which gave negative results with the *Xenopus laevis* test and positive results with the HI test were proved pregnancies between 16 and 20 weeks.

The HI test, being more sensitive, might be an aid to the early diagnosis of choriocarcinoma and hydatidiform mole, for by making further dilutions the system might be adapted so as to give a quantitative estimate of HCG levels.

The presence of non-specific agglutinins in a urine specimen can result in the production of an agglutination pattern at the 1:5 level in which case agglutination will also occur in the control tube (Fig. 2). Two specimens in this trial produced such a pattern; one of these showed agglutination at the 1:5 and 1:10 levels, but in both cases a positive result was recorded since inhibition of agglutination was observed in the higher dilutions of test and control suspensions. It will therefore be understood that preparation of adequate controls is of vital importance.

Apart from the above-mentioned results all other tests were observed to give positive patterns in all 3 dilutions.

Specificity in pregnancy testing is a prerequisite; dependability must be near to 100%, and with the new system used in this trial, which includes adequate controls, it would seem that false results are improbable. Medico-legal aspects and possible repercussions arising from the use of a not too satisfactory method require the closest examination and consideration.

SUMMARY

The paper deals with a comparative study of 107 specimens of urine submitted for the routine *Xenopus laevis* test at this Institute. The haemagglutination inhibition (HI) test was carried out in parallel. The accuracy obtained was as follows:

Xenopus laevis *test*		*HI test*	
Correct positive	85%	Positive	98%
Correct negative	100%	Negative	98%

It would appear that the HI method is at least as accurate as the *Xenopus laevis* test. The advantages and disadvantages of the two methods are outlined in the text.

I should like to record my appreciation to the Director of the South African Institute for Medical Research for facilities provided and to Miss F. E. Simpson of this Institute for technical assistance. I particularly want to thank Mr. F. Peché of Burroughs Wellcome, Johannesburg, for his valuable assistance in the follow-up of cases. I also wish to thank the many gynaecologists and general practitioners for their cooperation in supplying data for the confirmation of the diagnosis. Material used in this trial was kindly provided by Wellcome Research Laboratories, Beckenham, England.

REFERENCES

1. Cantarow, A. and Trumper, M. (1962): *Clinical Biochemistry*, 6th ed. Philadelphia: Saunders.
2. Lister, U. M. (1961): Practitioner, **186**, 590.
3. Smith, R. A., Albert, A. and Randall, L. M. (1951): Amer. J. Obstet. Gynec., **61**, 514-526.
4. Edward, A. (1961): *A Manual of Pregnancy Testing*, 1st ed. London: Churchill.
5. Weisman, A. I. and Coates, C. W. (1944): *The South African Frog (Xenopus laevis) in Pregnancy Diagnosis*. New York: New York Biologic Research Foundation.
6. Aschheim, S. and Zondek, B. (1928): Klin. Wschr., **7**, 1401.
7. Scott, L. D. (1940): Brit. J. Exp. Path., **21**, 320.
8. Fulthorpe, A. J., Parke, J. A. C., Torey, J. E. and Monckton, J. C. (1963): Brit. Med. J., **1**, 1050-1054.
9. Wide, L. (1962): Acta endocr. (Kbh.), **41**, suppl. 70.
10. Shapiro, H. A. and Zwarenstein, H. (1933): Proc. Roy. Soc. S.A., October 1933 (see Trans. Roy. Soc. S.A., 1934, 22, LXXV).