**Title:** IMPENDING OVULATION TEST

**Abstract**

Method for detecting impending ovulation in the human female by testing means employable by the average person. The test method involves contacting vaginal fluid samples with chemicals that indicate the presence of peroxidase in the vaginal fluid samples, e.g. a chromogenic substrate of peroxidase mixed with a hydroperoxide.
 Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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Description

Impending Ovulation Test

Technical Field
This invention relates to methods of detecting
impending ovulation in human females, including methods
which are sufficiently simple so that a woman can carry
out the test on herself without the aid of a physician.

Background Art
While prior methods of fertility or ovulation testing
have been proposed, prior tests have one or more unde-
sirable aspects. For example, in the prior art the
method of utilizing thermometry (the basal body temperature
method) provides information on fertility, but this
test merely indicates that ovulation has already occurred
and does not detect impending ovulation.

The prior ovulation detection methods involving
an examination of cervical mucus for its flow properties,
saline content, glucose content and the like, are also
deficient in that they do not easily lend themselves
to self-examination by the woman and require sampling
of portions of the vagina. Similarly, microscopic exami-
nation of vaginal cells for staining characteristics
and morphology require expensive apparatus and involve
techniques which are usually beyond the skill of the
average woman. Estrogen analysis of blood and urine
is likewise complicated and difficult to carry out.

Means for peroxidase testing have been known since
1898. Such means have been used for over 75 years for
the detection of blood, including commercial articles
sold, for example, by Smith Kline and French Laboratories
and by Miles Laboratories, for the detection of occult
blood in urine and feces. Despite the long history
of the peroxidase test it has not been previously used
to determine the surge in estrogen-induced peroxidase
in vaginal fluid for the detection of impending ovulation.
This may be due to the fact that pathological conditions
or injuries resulting in blood being present in the
vagina contribute to a false positive indication for
estrogen-induced peroxidase. It is known that hemoglobin
and its degradation products exhibit a peroxidase-like
reaction and, indeed, this property is utilized in the
occult blood test for urine and feces.

Disclosure of Invention
In the approximately two to three days prior to
ovulation in the menstrual cycle of normal human females
there is a surge in the amount of estrogen-induced peroxi-
dase in the vaginal fluid. A simple form of the invention
contemplates the woman taking a sample of vaginal fluid
with, for example, a moistened cotton swab and contacting
the swab with a fibulous material comprising a substance
which will cause a visible change in the presence of
peroxidase and a peroxide. Such visible change could
be a change in color or in luminescence. The finding
that the peroxidase is present in the vaginal fluid
makes it possible for a woman to readily obtain a sample
with a moistened cotton swab with no harm to herself.

One of the preferred forms of the test involves
the use of a paper comprising starch and an iodide salt
which produces a blue color in the presence of a peroxidase
when moistened with hydrogen peroxide. When peroxidase
is present in the vaginal fluid the aforesaid test sheet
will turn blue.

Some of the means used in this invention detect
both peroxidase and peroxidase-like substances, such
as hemoglobin and its degradation products.
Best Mode For Carrying Out the Invention

A simple method for the detection of impending ovulation in women, as in the present invention, is of the highest importance to the human race and is of paramount interest to such prestigious organizations as the Population Council, the Ford Foundation, the National Institutes of Health and the World Health Organization. With the present invention a woman can, by herself, determine her impending fertile time. There is extensive clinical evidence that for women the fertile time commences within 12 to 72 hours of ovulation (C. Tietze Fertility and Sterility, Vol. 11, p. 485, 1960). By abstaining from coitus or by otherwise protecting herself from insemination during the fertile time a woman can avoid pregnancy. Thus with the aid of the present invention which enables a woman to determine her fertile time, this form of birth control could, if practiced widely, substantially reduce the rate of world population growth. Using the present invention one may practice birth control without interference with the normal female hormonal function, such as occurs with the contraceptive pill which is objectionable to certain segments of the world population on religious grounds, as well as to others on medical grounds due to the possible serious side effects. A woman practicing abstinence during the fertile period as determined by the present invention may avoid the need for contraceptive devices, such as the intrauterine device, for birth control which are considered undesirable to some.

The present invention may also be an aid to couples who wish to have a child, but have failed because, for example, incorrect timing of coitus. Thus it may be seen that because the present invention can be a valuable aid in family planning, it serves an important humanitarian purpose.
A preferred embodiment of the present invention uses a chromogenic substrate which responds rapidly to the estrogen-induced peroxidase of the vagina in the presence of a peroxide, but responds much more slowly to the action of hemoglobin or its degradation products. Such chromogenic substrates are characterized by the fact that the rate constant (commonly designated $k_4^*$; see B. Chance, *Advances in Enzymology*, 1951, Volume 12, pages 153-180) for the reaction of the substrate with the peroxidase-peroxidide complex exceeds about $10^5$ moles$^{-1}$ seconds$^{-1}$. Substrates having such high values of $k_4^*$ include, for example, $p,p^1$-biphenol, hydroquinone and 0-phenylenediamine. It should be noted that values of $k_4^*$ for a given chromogenic substance may differ somewhat with the choice of the particular hydroperoxide employed.

One of the preferred embodiments involves impregnating a bibulous strip of paper with an inorganic peroxide such as hydrogen peroxide, sodium peroxide, barium peroxide, strontium peroxide, sodium perborate, and the like or an organic peroxide, such as methyl hydroperoxide, ethyl hydroperoxide, cumene hydroperoxide, dimethoxy dihydroperoxy hexane, and the like. Hydrogen peroxide may be considered both an inorganic peroxide and as a hydroperoxide. Many compounds, for example sodium peroxide, barium peroxide, strontium peroxide, sodium perborate, and the bis (l-hydroxyalkyl) peroxides generate hydrogen peroxide when moistened. Enzymatic reactions such as the action in air of L-amino oxidase on L-amino acids also generate hydrogen peroxide.

Chromogenic peroxidase substrates which may be employed in the present invention include the following substances:

1) Monoamines, such as aniline and its derivatives, orthotoluidine, para-toluidine, etc.;
2) Diamines, such as ortho-phenylenediamine, N,N dimethylpara-phenylenediamine, N,N diethyl-phenylenediamine, benzidine, 3,3', 5,5', tetramethyl benzidine, dianisidine, o-tolidine, etc.;

3) Phenols, such as phenol per se, thymol, ortho, meta and para-cresols, alpha-napthol, p,p-dihydroxybiphenyl, phloroglucinol and guaiacol;

4) Aromatic acids, such as salicylic, pyrocatechic and gallic acids;

5) Leucodyes, such as leucomalachite green (to produce malachite green) and leucophenolphthalein (desirably employed in a alkaline medium);

6) Colored dyes, such as 2,6 dichlorophenol indophenol;

7) Various biological substances, such as epinephrine, the flavones, tyrosine, dihydrophenylalanine (producing an orange-reddish color) and tryptophane. Other substances such as gum guaiac, guaiaconic acid, Nadi reagent (producing a bluish color), bilirubin (producing a greenish color), iodides (which produce a brown color and, if starch is present, produce a deep blue color which is much stronger than iodide alone).

Some of the substances may be most effectively used in combination rather than individually. For example, Nadi reagent is such a mixture, namely naphthol and p-phenylenediamine, which gives a better final color than the individual components. Another example is a mixture of 4-amino antipyrine and 1,7 dihydroxynaphthaline.

Many of the chromogens, notably benzidine and its derivatives give a more intense color if halogen ions, such as iodide and bromide ions, or if halogenoid ions, such as thiocyanate and selenocynate ions, are present.

One of the preferred embodiments of the invention comprises 3,3', 5,5' tetramethyl benzidine and potassium.
thiocyanate; this mixture yields an intense blue color in a positive test.

Substrates which change their fluorescence in the presence of a peroxidase and hydrogen peroxide include a loss of fluorescence of scopoletin or a production of fluorescence with, for example, dichlorofluorescin or homovanillic acid. Chemiluminescence is produced in the presence of peroxidases and hydrogen peroxide for the following typical substances, luminol, zinc tetra phenylporphyrine and the like.

The color forming substance may undergo color change, not as a result of the direct action of a hydroperoxide, but by mediation through another compound which is acted upon by a hydroperoxide and does not itself become highly colored. Examples of such color-forming or color-changing substances are:

1) Starch and potassium iodide to produce the characteristic starch-iodine purple which is stronger in color than iodide alone produces.

2) Mixture of a ferrous salt, such as ferrous ammonium sulfate, and tannic acid to produce a dark color.

3) Mixture of potassium iodide and 3,3', 5,5' tetramethyl-benzidine to produce a blue-black color stronger than either one alone.

4) Mixture of potassium thiocyanate and o-tolidine forming a blue color stronger than o-tolidine alone.

5) Mixture of potassium thiocyanate and p, p' biphenol forming a black color stronger than p,p' biphenol alone.

Hereinafter such mixtures will also be referred to as chromogenic substrates of peroxidase, even if the mixture does not undergo a color change as a result of the direct action of a hydroperoxide.
Example 1

A woman who has regular menstrual cycles donated vaginal samples daily starting on day 5 of her cycle, where day 1 is taken as the day when menstruation began.

The vaginal samples were obtained by the woman using a standard six inch cotton-tipped swab ("Puritan", Hardwood Products Co.). The cotton end was moistened with water and rolled gently on the wall of the anterior vagina. The vaginal sample on the swab was divided into six parts, each part of which was kept moistened.

One portion of the daily sample was contacted with commercially-available starch-iodide test paper (Precision Laboratories) and on the paper was placed a drop of dilute aqueous hydrogen peroxide solution having a concentration of 0.005%. A positive test was indicated by the formation of a strong blue color in about 2 minutes. A negative test was indicated if no color or only weak color was produced in about 2 minutes.

For the woman tested the test was negative for the samples of days 5 through 8, but on days 9 and 10 a strong blue color (i.e. positive color) was obtained. On this same woman tests of lutenizing hormone (LH) were taken on daily samples of her urine and it was found that a surge in LH occurred on day 12. She reported that her cervical mucus felt slippery (the Billing Test) at days 11 and 12 and that her basal body temperature (BBT) rose on day 14. The woman started to menstruate on day 27. The test for peroxidase herein described occurred at a time consistant with the other tests and therefore the positive peroxidase result anticipated ovulation; ovulation presumably occurring on or about day 13.
Example 2

A portion of daily vaginal sample as described in example 1 was dipped into a 0.01% aqueous solution of orthotolidine (made up by diluting a 1% ethanolic solution of orthotolidine with 0.01 molar phosphate buffer at pH 6.5) and then dipped into a 0.005% solution of hydrogen peroxide. Vaginal samples of days 5 through 8 gave no color (i.e. a negative test) but samples of days 9 and 10 gave a strong color (i.e. a positive test).

Here again the test presumably anticipated ovulation.

In place of ortho-tolidine a number of chromogenic substrates may be used, such as guaiac, p,p' biphenol or 3,3' 5,5' tetramethylbenzidine. Said o-tolidine, p,p' biphenol or tetramethylbenzidine solutions may also comprise a bromide, iodide or thiocyanate. Instead of solutions of the above chromogenic substrates, a bibulous mat, for example paper, comprising them may be employed. After contact with the swab comprising the vaginal sample the bibulous mat is moistened with hydrogen peroxide to produce the color indication if peroxidase is present.

One may also use dichlorfluorescein in place of ortho-tolidine but now a positive test is a strong yellow fluorescence (as observed under Wood's Lamp illumination) while no or only weak fluorescence is a negative test.

Example 3

A portion of the daily vaginal sample as described in example 1 was subjected to the commercially-available "Hematest" test for occult blood (Ames Company). The vaginal sample is applied to the bibulous white paper provided, to which a moistened pill containing, according to the manufacturer, ortho-tolidine, strontium peroxide, calcium acetate and tartaric acid is contacted. Vaginal samples of days 5 through 8 gave no color or only weak
color, but samples of days 9 and 10 gave a strong blue color. Here again the test presumably anticipated ovulation.

Example 4

A portion of the daily vaginal sample as described in example 1 was subjected to the occult blood test portion of the commercially-available "N-Multistix" (Ames Company). According to the manufacturer this portion of the test stick contains ortho-tolidine and cumene hydroperoxide. The test stick gave no or only weak coloration for vaginal samples of days 5 through 8, but gave a strong blue coloration for samples of days 9 and 10. Here again the test presumably anticipated ovulation.

Example 5

A portion of the daily vaginal samples as described in example 1 was subjected to the commercially-available "Hemoccult" occult blood test for feces (Smith Kline and French Laboratories). According to the manufacturer this consists of a bibulous paper impregnated with an ethanolic solution of guaiac and allowed to dry. The vaginal samples were contacted with this test paper and then a 0.1% ethanolic solution of hydrogen peroxide was added. The test paper gave no or only weak coloration for vaginal samples of days 5 through 8, but gave a strong blue coloration for samples of days 9 and 10. Here again the test presumably anticipated ovulation.

2. A method according to claim 1 in which said test for peroxidase produces a color change.

3. A method according to claim 1 in which said test for peroxidase produces a change in luminescence.

4. A method according to claim 1 where the test for peroxidase comprises treating a vaginal fluid sample with a chromogenic substrate of peroxidase and a hydroperoxide.

5. A method according to claim 4 where the test for peroxidase comprises contacting vaginal fluid with an inorganic peroxide.

6. A method according to claim 1 where the test for peroxidase comprises contacting the vaginal fluid sample with a bibulous mat comprising a chromogenic substrate of peroxidase.

7. A claim according to claim 1 where the test for peroxidase comprises contacting the vaginal fluid sample with a bibulous mat, said mat comprising a chromogenic substrate for peroxidase and a hydroperoxide.

8. A claim according to claim 5 where the test for peroxidase comprises contacting a vaginal fluid sample with a chromogenic substrate for peroxidase and an inorganic peroxide in a pill.
9. A claim according to claim 6 where the chromogenic substrate of peroxidase is guaiac.

10. A claim according to claim 7 where the chromogenic substrate for peroxidase is 0-tolidine and the hydroperoxide is cumene hydroperoxide.

11. A claim according to claim 8 where the chromogenic substrate for peroxidase is 0-tolidine and the inorganic peroxide is strontium peroxide.

12. A claim according to claim 4 where the vaginal fluid sample is absorbed on a bibuluous material which has been in contact with the vagina.

13. A claim according to claim 4 where the chromogenic substrate is starch and a soluble iodide salt.

14. A claim according to claim 4 where the chromogenic substrate is guaiac.

15. A claim according to claim 4 where the chromogenic substrate has a rate constant \( k_4 \) (as defined herein) in excess of about 100,000.

16. A claim according to claim 4 where the chromogenic substrate is \( p,p' \) biphenol.

17. A claim according to claim 4 where the chromogenic substrate is ortho-tolidine and a soluble iodide salt.

18. A claim according to claim 4 where the chromogenic substrate is \( 3,3',5,5' \) tetramethylbenzidine and a soluble iodide salt.
19. A claim according to claim 4 where the chromogenic substrate is 3,3',5,5' tetramethylbenzidine and a soluble bromide salt.

20. A claim according to claim 4 where the chromogenic substrate is 3,3',5,5' tetramethylbenzidine and a soluble thiocyanate salt.

21. A claim according to claim 4 where the chromogenic substrate is orthodianisidine and a soluble iodide salt.

22. A claim according to claim 4 where the chromogenic substrate changes its luminescence after oxidation by peroxide in the presence of peroxidase.

23. A claim according to claim 4 where the hydroperoxide is hydrogen peroxide.

24. A claim according to claim 4 where the hydroperoxide is cumene hydroperoxide.

25. A claim according to claim 4 where the chromogenic substrate is a mixture of p,p' biphenol and a soluble thiocyanate salt.

26. A method according to claim 12 where the bibulous mat which has been in contact with the vagina comprises a chromogenic substrate for peroxidase.

27. A claim according to claim 26 where the chromogenic substrate for peroxidase is guaiac.

28. A claim according to claim 26 where the chromogenic substrate for peroxidase is bilirubin.
29. A claim according to claim 7 where the chromogenic substrate is o-tolidine and the hydroperoxide is dimethoxy dihydroperoxy hexane.

30. A claim according to claim 1 where the test for peroxidase comprises contacting a vaginal fluid sample with a compound which generates hydrogen peroxide.

31. A claim according to claim 1 where the test for peroxidase comprises contacting a vaginal fluid sample with an enzyme-substrate system which generates hydrogen peroxide.
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. 0 G01N 33/00; C12Q 1/28
U.S. CL. 435/28,806; 23/230B,917

II. FIELDS SEARCHED

Minimum Documentation Sought

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III. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>CA.A, 854,156, PUBLISHED 20 OCTOBER 1970, FOSTER.</td>
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<td>X</td>
<td>N, CONTRACEPTION, ISSUED JUNE 1975, J.A. BLAIN ET AL, PEROXIDASE IN HUMAN CERVICAL MUCUS DURING THE MENSTRUAL CYCLE, PAGES 677-680.</td>
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- "O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search 1

01 AUGUST 1980

Date of Mailing of this International Search Report 2

13 AUG 1980

International Searching Authority 1

ISA/US

Signature of Authorized Officer 2

ROBERT J. WARREN

Form PCT/ISA/210 (second sheet) (October 1977)