

# Diagnostic Tests for Confirmation of Mating in Adult Female Wistar Rats: An Experimental Study

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## ABSTRACT

**Introduction:** The assessment of confirmation of mating in rats is a procedure which requires expertise with caution, and careful execution. Rats are spontaneous ovulators and are widely used to study the reproductive system.

**Aim:** The aim of the present study was to compare diagnostic tests for confirming mating in adult female wistar rats.

**Materials and Methods:** The present experimental research was duly approved by the Institutional Animal Ethical Committee and conducted at the Centre for Toxicology and Developmental Research (CEFTE) at Sri Ramachandra Institute of Higher Education and Research (Deemed to be University). The study was conducted in December 2019. A total of 72 adult Wistar rats (36 males and 36 females) were used in the study. To identify the event of mating, the rats were subjected to the following confirmatory methods: naked eye examination, non invasive

vaginal lavage method, and abdominal palpatory method. Data were subjected to statistical analysis using non parametric tests with SPSS software (version 25.0). The level of significance was set at  $p < 0.05$ . Comparison of all three methods was done using non parametric tests (post hoc with Wilcoxon signed-rank test).

**Results:** The non invasive vaginal lavage method was found to be more accurate (72.22%) compared to the visual method (16.66%) and the abdominal palpatory method (11.11%). A significant difference was seen when comparing the three methods using post hoc with Wilcoxon signed-rank test ( $p < 0.05$ ).

**Conclusion:** Based on the findings, it can be concluded that the vaginal lavage method is more specific and easy to confirm the occurrence of mating in adult female Wistar rats. The non invasive vaginal lavage method is a simple, easy, and accurate method for estimating pregnancy in cohabitating/generation studies.

**Keywords:** Cohabitation, Copulatory plug, Ovulator, Pregnancy test, Vaginal lavage

## INTRODUCTION

Reproduction is an essential process for a species, starting with the mating phase, followed by the initiation of day counting from day one. Rats are spontaneous ovulators and are genetically and biologically homogeneous compared to humans [1,2]. Therefore, rats are extensively used in biomedical research. The assessment of mating confirmation in rodents is a highly skilled technique conducted in laboratory animals [3]. Animals may undergo mechanical stress, either directly or indirectly, which might lead to spontaneous abortion.

Rats are commonly used as primary rodents for fertility and reproductive toxicological studies. They are also useful in the early diagnosis of pregnancy and studying foetal characteristics [4]. Estimating the oestrous cycle is a benchmark for the reproductive cycle in female rats. Pregnant rats are used as animal models for research in reproductive toxicology [5]. Due to technical difficulties, it is necessary to perform combined screening tests for better discrimination of direct effects on reproductive development [6].

Various methods like vaginal lavage (non-invasive), swab test, visual/naked eye examination, and abdominal palpation method, are available for confirming whether mating has occurred in rats [7]. Based on previous research, the vaginal lavage method has been found to be more specific and reliable for confirming mating in rats [8]. Hence, in the present study, different methods employed for confirming mating in laboratory rats, including vaginal lavage (non-invasive), visual/naked eye examination, and abdominal palpation method, were compared.

## MATERIALS AND METHODS

This experimental study was conducted in December 2019. In the present study, 72 adult Wistar rats (36 males and 36 females) were obtained from a CPCSEA-approved vendor (Invivo Bioscience,

Bengaluru) (Species: *Rattus Norvegicus*). The research was duly approved by the Institutional Animal Ethical Committee (IAEC/55/SRU/614/2018) at the Centre for Toxicology and Developmental Research (CEFTE), Sri Ramachandra Institute of Higher Education and Research (Deemed to be University).

**Sample size:** The researchers have presented preliminary confirmatory data on mating to ensure the fertility index, for which 36 male and 36 female adult Wistar rats were selected based on guidelines [9]. For any preclinical study [6-10], a certain number of animals are required for statistical analysis; hence, it was decided to go with six animals per group. Male and female rats were housed individually (i.e., three rats per cage) in polypropylene cages (47×34×18 cm) topped with a stainless steel grid and bedded with autoclaved sterile paddy husk (approximately 1 inch in height), which was replaced daily.

A normal circadian rhythm of 12:12 hours (dark:light ratio) was followed, maintaining a temperature of  $22 \pm 2^\circ\text{C}$  and a relative humidity of  $70 \pm 10\%$ . Throughout the experimental period, the animals were provided with bottled portable UV treated water (ad libitum) in autoclaved bottles, and standard pelleted rodent feed purchased from a vendor (rat and mice pellets, manufactured by: VRK Nutritional Solutions, Sangli-416416, Maharashtra) was provided [10]. Animals were handled using sterile gloved hands.

The entire work was done based on the guidelines proposed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

**Mating protocol:** At 6 pm, animals were left for cohabitation with the male partner, which was placed separately in Male:Female (M:F, 1:1) individual cages for mating [9]. The next day, before 8 am, copulation was checked around the vaginal area to determine whether mating had occurred, following the methods described below. All 36 female animals were subjected to tests a and b in the given chronology.

**Methods for assessment of copulation:**

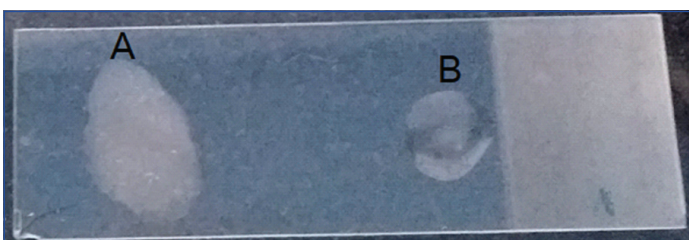
a) Visual method/Naked eye method: Female rats were gently scuffed on the dorsal surface using animal handling techniques. The vaginal orifice was examined for the presence of an occluded semen/copulatory plug [Table/Fig-1]. Deposition of fluid (seminal/prostatic) indicated confirmation of mating [11]. If no plug was observed, the same animal was subjected to the lavage method.

b) Non invasive vaginal lavage method: The female rat was scuffed in the left hand with the snout placed rostrally, and then the tail was lifted and looped in the 4<sup>th</sup> web space. Using the right hand, 200 µL of normal saline {0.9% Normal Saline (NS)} was aspirated through a sterile disposable pipette. The distal end tip of the sterile pipette was placed near the vaginal orifice on the lower ventral surface of the animal. Without penetrating the pipette tip deeply, the NS fluid was gently flushed to rinse/wash and aspirate the cells into the pipette [7].

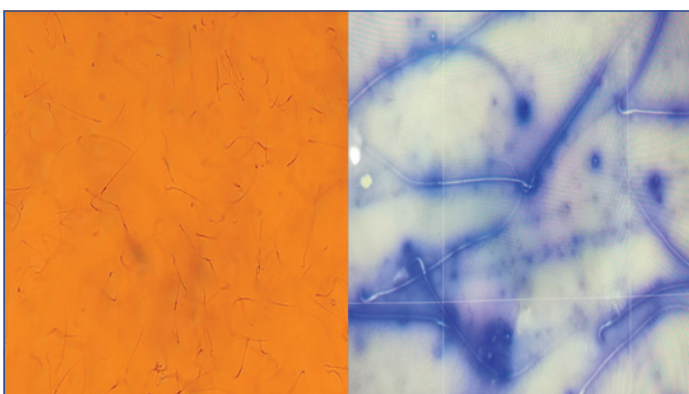


[Table/Fig-1]: Image showing copulatory plug using visual method.

The aspirated fluid was dropped (2 to 3 drops) and spread over a sterile glass slide [Table/Fig-2], then visualised under a light microscope at low power (10X) and low illumination. If mating had occurred, the presence of sperm could be directly visualised in the lavage cells at low power [Table/Fig-3a]. The structure of sperm could be observed using crystal violet stain [Table/Fig-3b]. If no sperm were observed in the aspirated lavage sample, the same animal was allowed to cohabitate with the same male partner.



[Table/Fig-2]: Smear showing vaginal lavage sample: a) presence of sperms in lavage sample (Turbid); b) absence of sperm and clear saline was found.



[Table/Fig-3]: Smear showing vaginal lavage sample: a) lavage sample containing sperms, b) slide containing sperms stained by crystal violet stain. (Images from left to right)

**Abdominal palpation method:** The rat was gently held with one hand from behind. The thumb of the other hand was placed on the middle third of the anterior abdomen, using three fingers on the ventral midline. The three fingers were pressed upwards and laterally in a gentle manner. The embryos could be palpated through the anterior abdominal wall between the thumb and the three fingers [12]. A veterinarian can confirm pregnancy using abdominal palpation between the 10<sup>th</sup> and 14<sup>th</sup> day.

At 8 am, female rats were subjected to the visual method to check for a positive sign of a vaginal plug, as shown in [Table/Fig-1]. If the visual method didn't indicate signs of mating, vaginal lavage was performed on the same animal as described earlier [7]. If neither method confirmed copulation, the animals were left to cohabitate with the same partner until the 10<sup>th</sup> day, after which the palpation method was done.

If female rats did not show a positive sign, the veterinarian was called on the 10<sup>th</sup> or 11<sup>th</sup> day to perform methods a and b and check for confirmation.

**STATISTICAL ANALYSIS**

The data was subjected to statistical analysis using SPSS software (IBM SPSS version 25.0). The level of significance was set at  $p < 0.05$ . A non-parametric test (post hoc with Wilcoxon signed-rank test) was used to compare all three methods.

**RESULTS**

A total of 36 male and 36 female Wistar rats were used in a 1:1 ratio. Female Wistar rats were used to confirm mating using naked eye examination, non invasive vaginal lavage, and abdominal palpation methods. The researchers observed that the non invasive vaginal lavage method was more accurate and easier to detect mating compared to the other two techniques (visual method and abdominal palpation method) [Table/Fig-4]. Out of the 36 rats, 26 (72.22%) showed a positive sign for mating, and pregnancy was confirmed accurately. Gross/naked eye examination showed six rats (16.66%) with a positive sign for mating and pregnancy confirmation, while only four rats (11.11%) tested positive using the abdominal palpation method. There was no induced mechanical stress, and no pseudo-pregnancy was observed.

S. No.	Methods	No. Wistar rats tested positive	Frequency %
1	Visual/Naked eye method	6	16.66%
2	Non invasive vaginal lavage method	26	72.22%
3	Abdominal palpation method	4	11.11%

[Table/Fig-4]: Frequency table showing mating confirmation methods (n=36).

We also observed a significant difference between the tests based on the post hoc with Wilcoxon signed-rank test. All three methods were found to be statistically significant ( $p \leq 0.05$ ) [Table/Fig-5].

	Vaginal lavage with visual method	Abdominal palpation with visual method	Abdominal palpation with vaginal lavage method
Z	-5.099 <sup>b</sup>	-2.000	-2.000
Asymp Sig. (2-tailed)	0.000**	0.05**	0.05**

[Table/Fig-5]: Statistical comparison of three methods used in the present study for mating confirmation.

\*\* $p \leq 0.05$ ; <sup>b</sup>: based on negative ranks

**DISCUSSION**

Rats are highly active nocturnally, so a 12-hour dark cycle was chosen for mating. Female rats reach sexual maturity between 8-12 weeks, while the breeding life of both sexes starts between 9-12 weeks [13]. The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and reproductive/developmental guidelines propose either a 1:2 or 1:1 ratio, with 1:2 possibly being used for regulated developmental findings, and 1:1 for fertility index and confirmation studies [9]. The present study achieved 100% success with a 1:1 ratio.

Female rats ovulate approximately 10-20 eggs every four to five days. The gestational period for female rats is 20-21 days. Therefore, rats at the age of 92 days were used to study the different methods of mating.

There are four methods for confirming mating in rats: the naked eye method, vaginal lavage method, abdominal palpation method, and cotton swab method.

The presence of a semen plug or copulatory plug over the external vaginal orifice showed that copulation has occurred. Upon observing the plug, the female rat was immediately separated from the male partner to prevent further copulation. The plug helps transport sperm into the rat's uterus [14]. However, it easily dislodges upon drying or when the animal moves around. In the present study, the author found that the visual method was more precise, as fresh copulatory plugs were detected within two hours of mating ( $p < 0.001^{**}$ ) using the Wilcoxon signed-rank test. The copulatory plug was observed in six females during the naked eye examination. The plug can be either vulvar or deep, and if it is deep, sterile tools and magnifying loupes are needed to confirm mating in mice [15]. It's important to note that untrained or unskilled individuals could potentially induce mechanical stress to the animals during evaluation or examination.

The vaginal lavage method was used to confirm mating. In this study, we observed that the vaginal lavage method was not only non invasive but also highly accurate and statistically significant ( $p < 0.05$ ), similar to the visual method and abdominal palpation method [8]. Skilled and trained personnel are required to perform this test. Additionally, mating could be assessed even after a few hours, even if the copulatory plug had been dislodged.

We also performed the abdominal palpation method, which was also found to be statistically significant ( $p < 0.05$ ). Although, all three methods mentioned above for confirming mating were highly accurate, the visual method was found to be more effective as we could visually observe the copulatory plug, which was statistically significant.

It has been documented that the use of a disposable sterile plastic headpiece otoscope can help confirm mating [16]. However, we did not use this method as it is invasive and could potentially induce mechanical stress. Additionally, using a fresh sterile otoscope for each animal would be expensive and require specific skills.

### Limitation(s)

The author were unable to identify the developing foetus before the 10<sup>th</sup> day of gestation using the abdominal palpation method for mating confirmation.

### CONCLUSION(S)

Based on the findings of the present study, it can be concluded that the non invasive vaginal lavage method accurately confirms mating compared to the visual and abdominal palpation methods in laboratory rats. The method is relatively simple in terms of time and skill requirements and is highly accurate without causing mechanical stress or trauma to the animal.

**Declaration:** The current research study is a part of the PhD work carried out by Mr. Yugesk K under the supervision of Dr. S. Senthil Kumar Sampath Kumar (Supervisor). IAEC approval was obtained for the entire PhD project, and a part of it is being published in this article.

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