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**TECHNICAL REPORT**

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# Lugol's Test Reexamined again: Buccal Cells*

**ABSTRACT:** Lugol's iodine staining technique was used to examine oral samples from 10 men and 10 women. Examination of saliva samples before and after extraction with water shows that the low levels (49 positive cells and 3,951 negative cells) of glycogen in buccal epithelial cells become even lower after water extraction (0 positive cells and 4,000 negative cells). In addition to the 20 samples used in this paper, 40 oral swabs extracted with water were examined under classroom conditions with much less than 1% of the epithelial cells being positive for glycogen. Furthermore, 119 saliva samples from chewed gauze in sexual assault kits were extracted with water and all of them yielded less than 1% glycogen positive cells. This paper proposes that when more than 1% of the nucleated squamous epithelial cells are glycogen positive with Lugol's test after extraction in water, it is reasonable to eliminate the mouth as a source of these glycogen positive cells.

**KEYWORDS:** forensic science, forensic biology, body fluid identification, vaginal cells, buccal cells, Lugol's iodine staining technique, glycogen in vaginal cells, nucleated squamous epithelial cells, epithelial cells, glycogen in buccal cells

Lugol's test (Lugol's iodine staining technique) is an iodine-based test for glycogen in nucleated squamous epithelial cells. It has been used to identify vaginal epithelial cells. From 1924 (1,2) to present, it has gone through several cycles of acceptance and rejection. The acceptance is based on the very high glycogen content of vaginal epithelial cells from menarche to menopause (1–7) and the rejection of the test is based on the presence of glycogen containing nucleated squamous epithelial cells from the urethral meatus of the penis (1,2,7–10) and the mucous lined membranes of the mouth (3,8). This paper will address the presence of glycogenated epithelial cells in the mouth. Two references allude to the fact that nucleated buccal epithelial cells containing glycogen can be confused with glycogen positive cells from the vagina. Randall (3) used Periodic Acid Schiff's reagent to identify 32 glycogen positive oral samples and 10 negative samples using female volunteers. Hausmann et al. (8) used Lugol's iodine to find 18 positive and 29 negative oral samples using male volunteers. One of the authors (ELJ) of this paper has presented the use of the Lugol's test to several forensic workshops and classes. One of the experiments in recent classes is for the participating forensic scientists to collect their own buccal cells on cotton swabs and allow them to dry overnight. The next day the buccal cells are extracted with water, mechanically shaken, transferred to a microscope slide, dried and examined with Lugol's iodine. To familiarize the forensic scientists with the Lugol's iodine test, they are given trays of slides that includes seven known vaginal samples and five known saliva samples. One of the known saliva samples is weakly positive and they are encouraged to find that sample before looking at the class saliva samples. None of the more than 40 participating forensic scientists performing this experiment has reported the presence of more than one Lugol positive (chocolate brown) cell. One of the authors (ELJ) prepared and examined the known saliva samples from each class and workshop to ensure that there were no lugol positive samples greater than 1%. It has also been observed in case work that out of 119 chewed gauze pads collected in sexual assault kits (predominately female samples), only seven showed Lugol positive results when extracted with water and mechanical shaking (11). All seven of these Lugol positive saliva samples had considerably less than 1% positive glycogen containing nucleated squamous epithelial cells. This paper is an attempt to illuminate why the published literature reports glycogen containing cells in the mouth and to propose that when more than 1% of the nucleated squamous epithelial cells from a questioned sample are Lugol positive after a water extraction, then the mouth can be eliminated as a source of those glycogen positive cells.

## Materials and Methods

Ten adult male and ten adult female volunteers gave oral samples on cotton swabs. A set of 20 microscope slides were prepared with the damp oral swabs by smearing them on a glass microscope slide. The oral swabs were dried at room temperature and extracted with water using a mechanical shaker (vortex) and another slide was prepared with this water extract. The water extract was allowed to dry with the aid of a heat lamp (less than 65°C) or at room temperature. Lugol's iodine (stock solution) is prepared by dissolving 10 g of potassium iodide and 5 g of iodine in 100 mL of water. The working solution of Lugol's iodine is 5% of the stock solution (one drop of Lugol stock solution and 19 drops of water) (4). The author (ELJ) has observed that the stock solution stored in a brown bottle

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will remain good for several years at room temperature and that the working solution is good for one day in an open test tube at room temperature. A sufficient quantity of Lugol's working solution is applied to an appropriate sized cover slip and laid down on the sample. The reaction of iodine with glycogen is instantaneous and the sample can be read immediately. A glycogen positive nucleated squamous epithelial cell must show a chocolate brown color in the body of the cell (cytoplasm) with a clear round to oval shaped unstained nucleus located somewhat centrally in the cytoplasm. A known vaginal sample that is strongly glycogen positive can be used as a positive control over and over. The stain is temporary, i.e., the brown color washes away with water and the slide can be air dried and it is ready to be used as a positive control again. Hausmann et al. (8) shows photographs of Lugol positive vaginal cells, Lugol positive penile cells and a Lugol positive oral cell. That Lugol positive oral cell is not what is typically seen when examining oral samples with Lugol's iodine. We found one cell out of the more than 4,000 oral cells examined in this study that was stained deep brown like that cell. Glycogen negative squamous epithelial cells will remain unstained with a slight yellowish color and their nuclei will be difficult to observe with normal brightfield illumination. Keratinized squamous epithelial cells (anucleated) are found on the palate (roof of the mouth) and are a natural part of saliva (12). They also make up the outer layer of our skin and are commonly found on penile, breast and skin swabs. These keratinized cells were not counted. When the microscope is properly set up to look for color with brightfield illumination, these cells are practically invisible. Using an opaque object to block part of the light beam below the substage condenser and above the field diaphragm (oblique transmitted illumination) or phase contrast will help in identifying the nucleus in an unstained cell. When oblique illumination or phase contrast is employed, the microscope is no longer set up for brightfield color examination therefore, the oblique illumination or phase contrast should be removed before searching for more Lugol positive cells. A total cell count of 200 was used to establish a negative sample or the percentage of glycogen positive cells. Because all of the oral samples contained more than 200 cells, a search of the slide was continued until a Lugol positive cell was identified. Any of the six samples reporting the presence of one positive cell (see Table 1) could have been negative if the cell count was started at random. Most of these samples contained in excess of a thousand cells, meaning that if the counting was done at random and stopped at 200 cells, then most of the samples reported with one lugol positive cell would have been negative.

TABLE 1—*Number of Lugol positive cells before water extraction (from a total of 200 buccal cells applied directly to a microscope slide from a wet oral swab) and after water extraction (from a total of 200 buccal cells applied to a microscope slide from a dried oral swab extracted with water).*

| Male Sample Numbers | Lugol Positive Cells before Extraction | Lugol Positive Cells after Extraction | Female Sample Numbers | Lugol Positive Cells before Extraction | Lugol Positive Cells after Extraction |
|---|---|---|---|---|---|
| 1 | 1 | 0 | 11 | 0 | 0 |
| 2 | 2 | 0 | 12 | 3 | 0 |
| 3 | 0 | 0 | 13 | 1 | 0 |
| 4 | 3 | 0 | 14 | 10 | 0 |
| 5 | 8 | 0 | 15 | 3 | 0 |
| 6 | 0 | 0 | 16 | 1 | 0 |
| 7 | 0 | 0 | 17 | 3 | 0 |
| 8 | 1 | 0 | 18 | 1 | 0 |
| 9 | 1 | 0 | 19 | 5 | 0 |
| 10 | 6 | 0 | 20 | 0 | 0 |
| | 22 Total 1.1% Average | 0 Total 0.0% Average | | 27 Total 1.4% Average | 0 Total 0.0% Average |

## Results and Discussion

Seven of ten males and eight of ten females had Lugol positive cells from oral samples applied directly to a slide. Four of ten males and five of ten females had more than one Lugol positive cell (See Table 1). The strongest positive samples (sample numbers 5 and 14) showed 18 light brown positive cells out of 400 for a 4.5% positive ratio. Both samples when extracted with water, mechanically shaken (vortexed) and dried on a slide yielded zero Lugol positive cells. The remaining 18 samples that were dried on cotton swabs and extracted with water also failed to reveal any glycogen positive nucleated squamous epithelial cells. One oral sample not reported in Table 1, gave one deep brown Lugol positive cell and 5 light brown Lugol positive cells before extraction (this same cell was previously described in Materials and Methods). This sample and several others were repeated or replaced because the water extract did not yield the required 200 cells. All samples gave more than 200 cells when the damp oral swab was rubbed on a glass slide.

Lugol's test for the identification of vaginal epithelial cells is based on the fact that the vast majority of vaginal samples collected from adult females have significant quantities of glycogenated nucleated squamous epithelial cells. Peabody et al. (4) reported 3 female volunteers who gave 24, 16 and 23 vaginal swabs throughout a menstrual cycle. All samples were lugol positive with the lowest percentage of Lugol positive cells being 10% positive. The graphs from this paper show that all three women average more than 25% Lugol positive cells. From 425 vaginal samples representing 208 cases from the author's (ELJ) casework (most victims had a vaginal aspirate, an introitus swab and/or a posterior fornix swab), 408 were Lugol positive and 17 were Lugol negative. Many of the 17 Lugol negative samples were either preadolescent or postmenopausal. All of the swab samples were prepared using water, mechanical mixing and drying the sample on a slide with mild heat (less than 65°C) or at room temperature. Several more of the 17 Lugol negative vaginal samples were recorded with positive samples from the same victim. Sometimes a vaginal sample did not contain a large enough number of nucleated squamous epithelial cells and this could be the reason why some victims had both Lugol positive and Lugol negative results in the same case. The author (ELJ) observed that the vaginal positive cells stain a deep chocolate brown with Lugol's iodine while most of the Lugol positive cells from oral samples stain a light brown color. In 20 oral samples, most fields of view did not show any Lugol positive cells while two fields of view at 200X showed two Lugol positive cells.

In forensic casework, it is sometimes important to establish the somatic origin of a sample. This is especially true in cases of vaginal rape with a foreign object (3,13). A vaginal sample and an oral sample are difficult to differentiate microscopically. Both the oral cavity and the vagina are lined with stratified nucleated squamous epithelial cells that consist of superficial, intermediate and parabasal cells. Both environments have similar micro-organisms such as bacteria and yeast. With Lugol's iodine test, vaginal and oral samples are relatively easy to tell apart most of the time. A vaginal sample can be Lugol negative and an extracted oral sample can contain a few Lugol positive cells. From a forensic serology standpoint, the oral sample will have elevated amylase levels while the vaginal sample will show lower levels or an absence of amylase activity (15).

Radial diffusion on agar gel with starch is one of the techniques for identifying the oral amylase (16) and works on the principal that amylase from saliva will break up the starch into oligosaccharides (limit dextrins), maltose and glucose (6). This reaction is observed by staining the starch gel with iodine solution (Lugol's working solution). Amylase positive areas are depicted as clear circles on a blue background. Glycogen is similar to starch in that it is a complex carbohydrate made up of glucose monomers joined together by an alpha 1-4 linkage (7). Starch is used by plants to store energy and glycogen is used by animals to store energy (8). Glycogen has more branching than starch and will stain chocolate brown while starch which has longer straight chains will stain blue with iodine (9). Both glycogen and starch will be broken down to oligosaccharides (limit dextrin), maltose and glucose by salivary amylase (10).

The cell membrane of nucleated squamous epithelial cells will not allow the amylase inside to breakdown the glycogen inside the cell. This is deduced from the fact that infant oral samples have both amylase in their saliva and high levels of glycogen in their buccal cells (8). The impermeability of the membrane of a nucleated squamous epithelial cell can be shown experimentally by treating a strong glycogen positive vaginal sample with salivary amylase. It will resist digestion for hours at 37°C. Normally, the salivary amylase will digest this quantity of glycogen in minutes (18). Oral samples when applied directly to a slide will sometimes show low levels of glycogen in their epithelial cells (3,8). A theoretical mechanism which explains the lowering of these glycogen levels in extracted saliva samples is that: when saliva samples are allowed to dry out on a swab, the concentration of amylase is increasing while the cellular membranes are probably stressed and/or broken. Upon extraction with water, these stressed and/or broken membranes will allow the rehydrated amylase to have access to the low levels of glycogen. This creates a sample with significantly less than 1% glycogen positive cells.

Randall (3,5) used the Periodic Acid Schiff stain (PAS) with a hematoxylin counter stain to detect the presence of glycogen in nucleated squamous epithelial cells in his papers establishing the usefulness of glycogen in identifying vaginal material. Houde, et al. (14) determined that the PAS stain would give similar results to Lugol's test for the detection of glycogen. He also observed that the PAS stain was more sensitive and less specific for glycogen than the Lugol iodine stain. In support of this observation, the author (ELJ) observed that clearly Lugol negative cells in both oral and vaginal samples would react with PAS to give a positive result. Randall's experiment (3) called for oral insertion of plastic tampon inserter shields that were swabbed with a water moistened cotton swab that was immediately smeared onto a glass slide. This paper's results of eight positive saliva samples out of ten women is in line with or lower than Randall's 32 positive oral samples out of 42 women. Three of the eight positive saliva samples in this study have one Lugol positive cell. In a different experiment, Randall (3) describes how ten volunteers spit directly on a microscope slide giving dried pools of saliva which averaged 4.7% PAS positive cells (range 1% to 9%). The 9% positive PAS sample is the highest percentage of glycogen positive cells reported from an oral sample. The authors surmise that this spitting technique of collecting oral samples would show several glycogen positive cells in the same field of view. In actual casework, a sample of dried saliva would be collected on a damp cotton swab, dried and brought back to the laboratory for extraction and examination. The authors believe that these spitting samples would produce less than 1% Lugol positive cells if they were collected with a water moistened swab, dried and then extracted with water and dried down on a slide for staining with Lugol's iodine.

Hausmann and Schellmann (8) used Lugol's iodine to identify glycogen positive cells in oral samples. Even though explicit details of sample preparation were not available in that paper, his results of 18 positive saliva samples out of 47 men is in line with or lower than this paper's results of seven positive saliva samples out of ten men. Three of the seven positive saliva samples from this paper have only one Lugol positive cell.

## Conclusions

It has been shown that the low levels of glycogen in the nucleated squamous epithelial cells of the mouth become even lower when the saliva sample is dried on a cotton swab (or cotton gauze), then extracted with water and dried on a microscope slide for examination with Lugol's iodine. With this technique, it has been shown that all oral samples tested to date yield less than 1% Lugol positive cells. The 20 oral swabs collected in this study have been supplemented with 40 oral swabs from classroom experiments and 117 saliva samples from casework. This paper shows why Lugol positive (8) and PAS positive cells (3) were reported from the mouth and that strong Lugol positive samples do not come from the mouth.

Even without water extraction and ignoring this paper's conclusion, the value of 10% positive Lugol exceeds the highest reported oral sample. Most vaginal samples are above 10% Lugol positive (4). The highest reported sample (9% PAS positive) was collected by spitting directly on a microscope slide then staining with PAS which is known to be less specific and more sensitive than Lugol's iodine (11). If you were to collect a questioned sample from a nonabsorbent surface with a damp swab and rub it directly to a microscope slide, then this higher value of 10% Lugol positive cells will be safe to use to eliminate the mouth as a source. However, if you allow this same swab to dry, then extract with water and transfer the water to a slide and dry it down, the 1% Lugol positive number can then be used to eliminate the mouth as a source.

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