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# Cytodiagnosis of Oral Squamous Cell Carcinoma in an Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*): Papanicolaou Staining and Immunocytochemistry Using Liquid-based Cytology

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**Abstract**

Although oral cytology using Papanicolaou staining may be useful for the early detection of oral premalignant lesions and squamous cell carcinoma (SCC) in humans, little work has been conducted on this topic in the veterinarian area. This paper describes the cytological features of oral cytology using Papanicolaou staining and immunocytochemistry on liquid-based cytology slides in a case of oral SCC in an Indo-Pacific bottlenose dolphin. Dysplastic cells with koilocytosis and SCC cells were identified using the Papanicolaou staining, and these cells were positive for p53 via the immunocytochemistry analysis. Therefore, our cytodiagnosis was SCC. We believe that the early detection of oral premalignant lesions and SCC in dolphins can be enormously improved with oral cytology using liquid-based cytology, Papanicolaou staining, and immunocytochemistry.

Keywords: bottlenose dolphin, liquid-based cytology, oral squamous cell carcinoma, Papanicolaou staining, p53 immunocytochemistry

Human papillomavirus (HPV) has been reported to be etiologically associated with oral squamous cell carcinoma (SCC) in humans.[1] Similarly, oral SCC thought to be caused by papillomavirus has been reported in bottlenose dolphins; however, there are few reports on oral cytology in such cases.[2,3] Although the gold standard for diagnosing oral SCC is the histopathology of biopsy or surgical specimens, cytology has the advantage of being minimally invasive and can be used to test a wide area. Therefore, oral cytology in humans is suitable for screening tests and is useful for the early detection and management of oral dysplasia and SCC.[4] Recently, research has been conducted in humans to determine whether liquid-based cytology (LBC) developed for cervical cytology can be applied to oral cytology.[4,5] The primary LBC methods are the SurePath method and the ThinPrep method, both of which have reported advantages over conventional methods, including a better cell recovery rate, cell preservability, and reduction of inadequate samples.[4,6] Additionally, LBC allows for immunocytochemistry and HPV genetic tests using the residual material left over after cytomorphological examinations.[4,7] The intranuclear accumulation of p53 protein that results from p53 gene mutation can be detected by immunocytochemistry, and it is often observed in human oral dysplasia and SCC. Moreover, p16 immunocytochemistry is regarded as a surrogate biomarker of high-risk HPV infection in humans.[8]

77 Veterinary cytology primarily uses the Giemsa (also known as Romanowsky or
78 Wright) stain.[9] Although Giemsa staining is excellent for observing hematological
79 tumor cells and utilizes a simple staining procedure, the cytomorphology cannot be
80 observed when the smear is thick or the cells are clustered.[9,10] Conversely, since the
81 Papanicolaou stain was developed to detect dysplasia and SCC of the uterine cervix,
82 it is excellent for observing intra-nuclear structures, keratinized cytoplasm, and cell
83 clusters.[10]

84 To our knowledge, this report is the first case of oral SCC in an Indo-Pacific
85 bottlenose dolphin diagnosed by oral cytology using LBC, Papanicolaou staining, and
86 immunocytochemistry. The purpose of this report was to describe not only the
87 cytomorphology of oral SCC but also the procedure and usefulness of LBC.

88 An over 45-year-old captive male Indo-Pacific bottlenose dolphin (Tursiops
89 aduncus) was reared in Okinawa Churaumi Aquarium (Japan) with a history of a
90 hemorrhagic ulcered mass on the right buccal mucosa that grew over a 2-year period.
91 Therefore, cytology and biopsy were performed for diagnosis.

92 The oral lesion was scraped with a toothbrush to collect cells for the cytologic
93 analysis, and the cells were immediately immersed and stirred in a large test tube
94 containing 20 mL of CytoRich™ Red Preservative Fluid (Becton Dickinson, Franklin
95 Lakes, NJ). LBC of the above sample was prepared using a modified SurePath

method (Becton Dickinson) (Table 1) (Fig. 1). These three SurePath slides were stained with Papanicolaou stain, p53, and p16 immunocytochemistry. In p53 immunocytochemistry, antigen retrieval was performed with citrate buffer (pH 6.0) heated at 90°C for 20 min, and a commercially available mouse primary antibody against p53 protein (clone DO-7, dilution 1:200) (Dako, Glostrup, Denmark) was used. The p16 immunocytochemistry was performed using a CINtec p16 kit (Roche Diagnostics GmbH, Mannheim, Germany).

The Papanicolaou stain slide was a moderate amount of cells and displayed an even cell distribution, with neutrophils and necrotic debris in the background. The air-dry artifact was not evident in this slide, and there were few erythrocytes even though the sample was bloody. The atypical cells appeared as dysplastic cells with koilocytosis (Fig. 2), and keratinized and non-keratinized SCC cells were present. Although the dysplastic cells and non-keratinized SCC cells mainly formed cell clusters, the keratinized SCC cells tended to appear individually. The non-keratinized SCC cells appeared as an overlapping structure (three-dimensional) cluster formation (Fig. 3), whereas the dysplastic cells displayed a sheet-like structure (two-dimensional). The keratinized SCC cells displayed a polygonal or fiber-like morphology (Fig. 4) and occasional onion-like clusters (keratin pearl) (Fig. 5). The nuclei of these atypical cells were generally mononuclear and round or oval shape,

but bi-nuclear and irregular shapes were also observed. The intra-nuclear features of the dysplastic and non-keratinized SCC cells were commonly characterized by fine or granular nuclear chromatin and a few prominent eosinophilic nucleoli. According to the immunocytochemistry analysis, dysplastic cells with koilocytosis and keratinized and non-keratinized SCC cells were positive for p53 (Fig. 6) but negative for p16. Based on the above cytomorphologic and immunocytochemical findings, our cytodiagnosis was SCC.

A histologic examination of the biopsy displayed nests of invasive cancer cells with abnormal keratinization. Based on the above histological findings, invasive SCC was diagnosed.

In the present case, dysplastic cells with koilocytosis and keratinized and non-keratinized SCC cells were observed. These cytological findings are consistent with the histological findings of oral papilloma and SCC in Atlantic bottlenose dolphins.[2] Koilocytosis is a cytomorphological feature characterized by a clear area around the nucleus and is considered the viral cytopathic effect of a papillomavirus infection.[11] In humans, koilocytic cells show a slightly increased N/C ratio, mono- or bi-nucleus, granular nuclear chromatin, and basophilic nucleoli. Meanwhile, in the present case, the nucleoli were larger than those in humans and were eosinophilic. Chun et al reported that more prominent nucleoli were present on the tumor cells on

the SurePath slides compared with the conventional method slides.[12] Therefore, the large nucleoli may be a result of the SurePath method although it is not evident whether the eosinophilic nucleoli of koilocytic cells are a characteristic of dolphins or a result of the staining conditions. To our knowledge, there are no previous reports of koilocytic cells in veterinary cytology, potentially because Papanicolaou staining has not been widely used in veterinary cytology, which made it impossible to observe detailed cytomorphology. Oral SCC in humans is considered to display a multistep process of development from premalignant lesions such as papilloma and leukoplakia.[13] We inferred that detecting these premalignant lesions with cytomorphology using Giemsa staining is difficult because the cell atypia of these lesions is likely mild in dolphins as well. Thus, it is possible that oral cytology using Papanicolaou staining leads to the early detection of oral premalignant lesions and SCC in dolphins.

SurePath and ThinPrep are the primary LBC methods approved by the Food and Drug Administration. Since SurePath can also be used manually as long as a slide rack and centrifuge are available, it can be performed in veterinary hospitals. The SurePath method principally functions through gravity sedimentation and electrical adhesion. Briefly, the settling chamber is clamped to a positively charged SurePath slide, and a cell suspension treated with CytoRich RED is added. Gravity then settles

153 the cells on the slide, and a positive-charged slide is combined with negatively
154 charged cells.[6] In the present case, air-dry artifacts were not present on the SurePath
155 slide because the cell collected toothbrush was immediately immersed in CytoRich
156 RED. Additionally, there were few erythrocytes on the SurePath slide despite
157 bleeding at the time of cell collection because the erythrocytes were hemolyzed by
158 CytoRich RED. Therefore, the SurePath slide can improve the slide quality (no air-dry
159 artifacts and a clear background). Moreover, this method provides a standardized
160 protocol, and the residual material left over after Papanicolaou staining can be used
161 for immunocytochemistry and a papillomavirus genetic test.[4]

162 In humans, HPV infection, chronic smoking, alcohol consumption, and periodontal
163 disease are established risk factors for oral SCC.[1] More than 100 types of HPV have
164 been identified in humans, including HPV types 16 and 18, which are known to be
165 strongly associated with the carcinogenesis of oral and cervical SCC.[1] HPV infection
166 within the oral cavity could result from oral sex.[14] Conversely, although only a limited
167 number of sequenced and characterized papillomavirus genomes have been
168 reported in dolphins, multiple types of papillomavirus have been identified in genital
169 and esophageal lesions in cetaceans.[15-17] Additionally, papilloma and SCC were
170 simultaneously observed in three free-ranging Atlantic bottlenose dolphins.[2] These
171 findings suggest that progressive pathologic processes involving the malignant

172 transformation of papillomavirus oral cavity infection can occur in dolphins. Although
173 the causes of oral papillomavirus infection in dolphins are uncertain, a recent study
174 reported that novel papillomavirus was detected in the feces of a wild bottlenose
175 dolphin.[18] Therefore, oral papillomavirus infection may be facilitated by the ability of
176 the virus to maintain its viability in aquatic environments.
177 The immunocytochemistry of p53 was reported to potentially be able to detect early
178 alterations associated with oral carcinogenesis in humans.[5] In this study, not only
179 SCC cells but also dysplastic cells of premalignant lesions were positive for p53.
180 Therefore, the immunocytochemistry of p53 is likely to be useful for detecting
181 premalignant lesions with slight cell atypia, which may be overlooked in visual
182 observation. Cervical and oropharyngeal SCCs in humans have reported a high
183 positive rate of p16 immunostaining (100% and 90.2%, respectively) .[8,19] The E7
184 oncoprotein of HPV binds to Rb and degrades it, and the oncogenic stress of the loss
185 of Rb function leads to the overexpression of p16. Therefore, positive p16
186 immunostaining is a well-accepted surrogate biomarker of transcriptionally active
187 HPV in the context of the cervix and oropharynx SCC.[8,19] However, in the present
188 case, all dysplastic cells and SCC cells were negative for p16 in immunostaining. This
189 discrepancy is likely caused by the currently identified cetacean papillomavirus lack
190 the E7 gene.[15-17,20] Thus, p16 immunostaining is not likely to detect papillomavirus in

oral premalignant lesions and SCC of cetaceans, including dolphins.

In conclusion, oral cytology using the SurePath method, Papanicolaou staining, and p53 immunocytochemistry is a useful diagnostic method and a promising tool for early detection, management, and research of oral SCC in dolphins.


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## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


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## Figure legends

Figure 1. SurePath manual method kit. A positively charged slide (arrow) and a settling chamber (arrowhead) set in a slide rack (circle).

Figure 2. The dysplastic cells with koilocytosis (arrow) appear as a sheet-like structure. Papanicolaou staining.

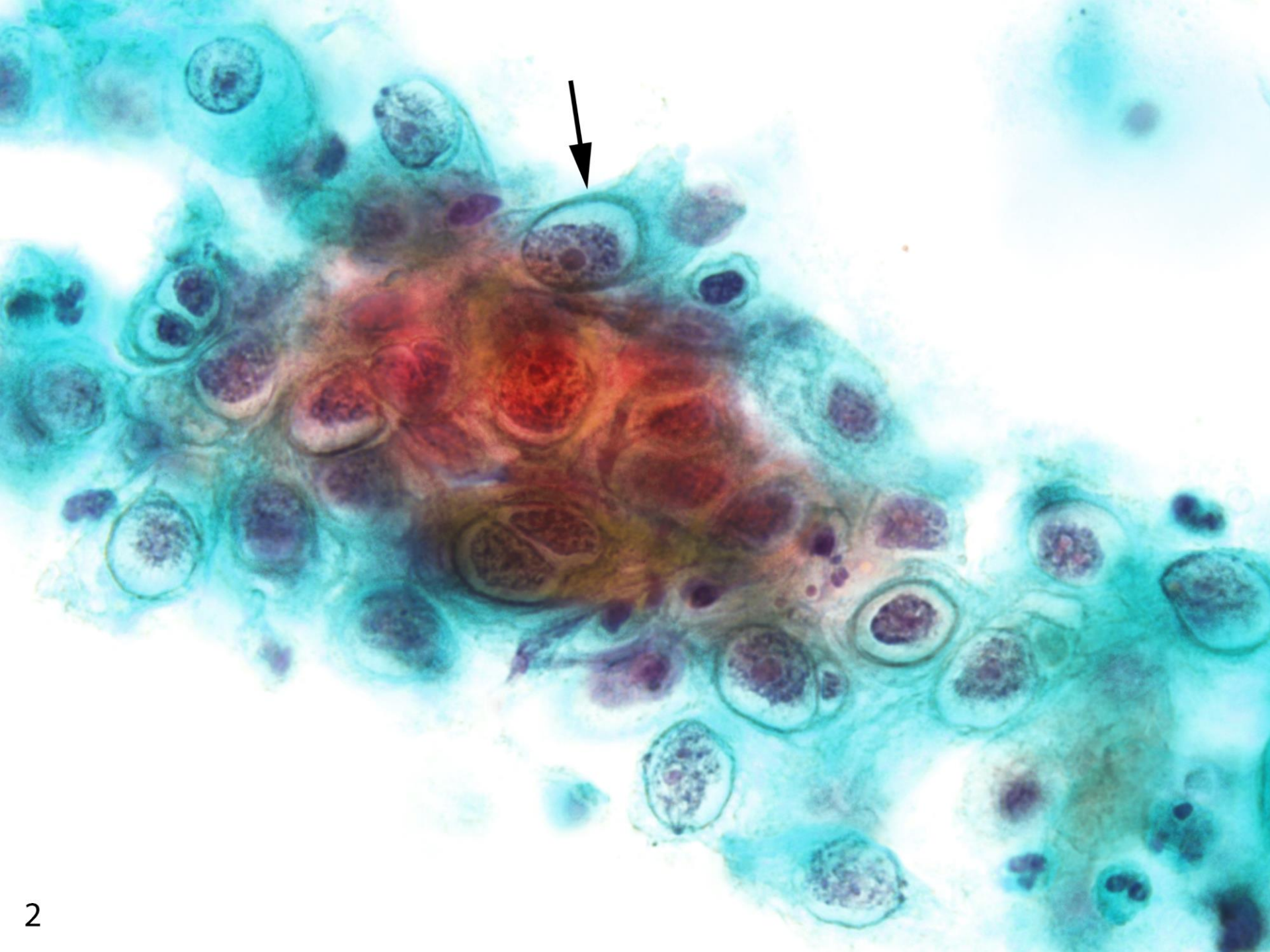
Figure 3. The non-keratinized SCC cells appear as overlapping structures. Papanicolaou staining.

Figure 4. The keratinized SCC cells displayed a polygonal morphology. Papanicolaou staining.

Figure 5. Occasionally, the keratinized SCC cells showed an onion-like cluster. Papanicolaou staining.

Figure 6. The SCC cells showed intense nuclear reactivity to p53 protein. Immunocytochemistry.

BD SurePath™
BD SurePath™
1



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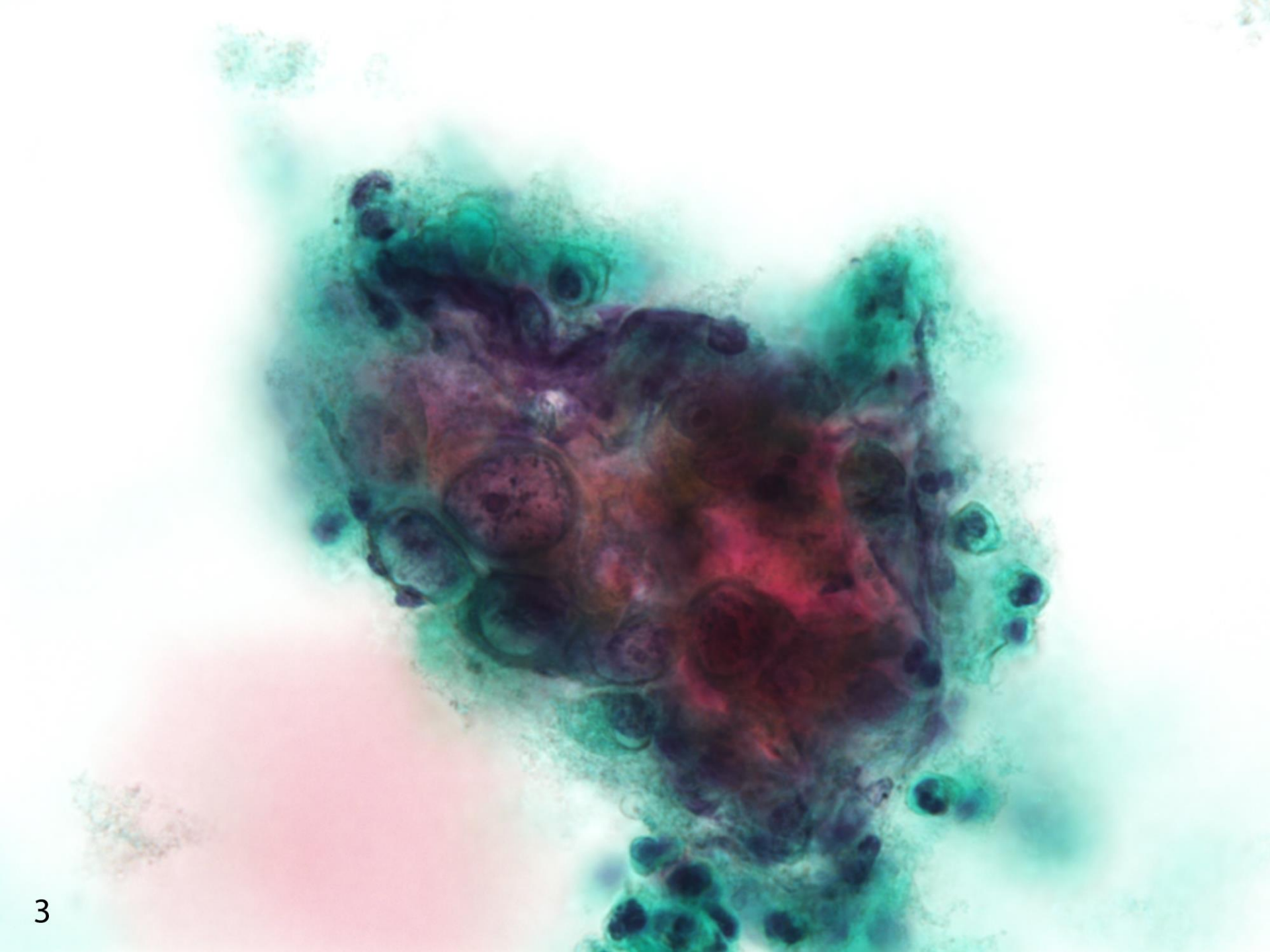

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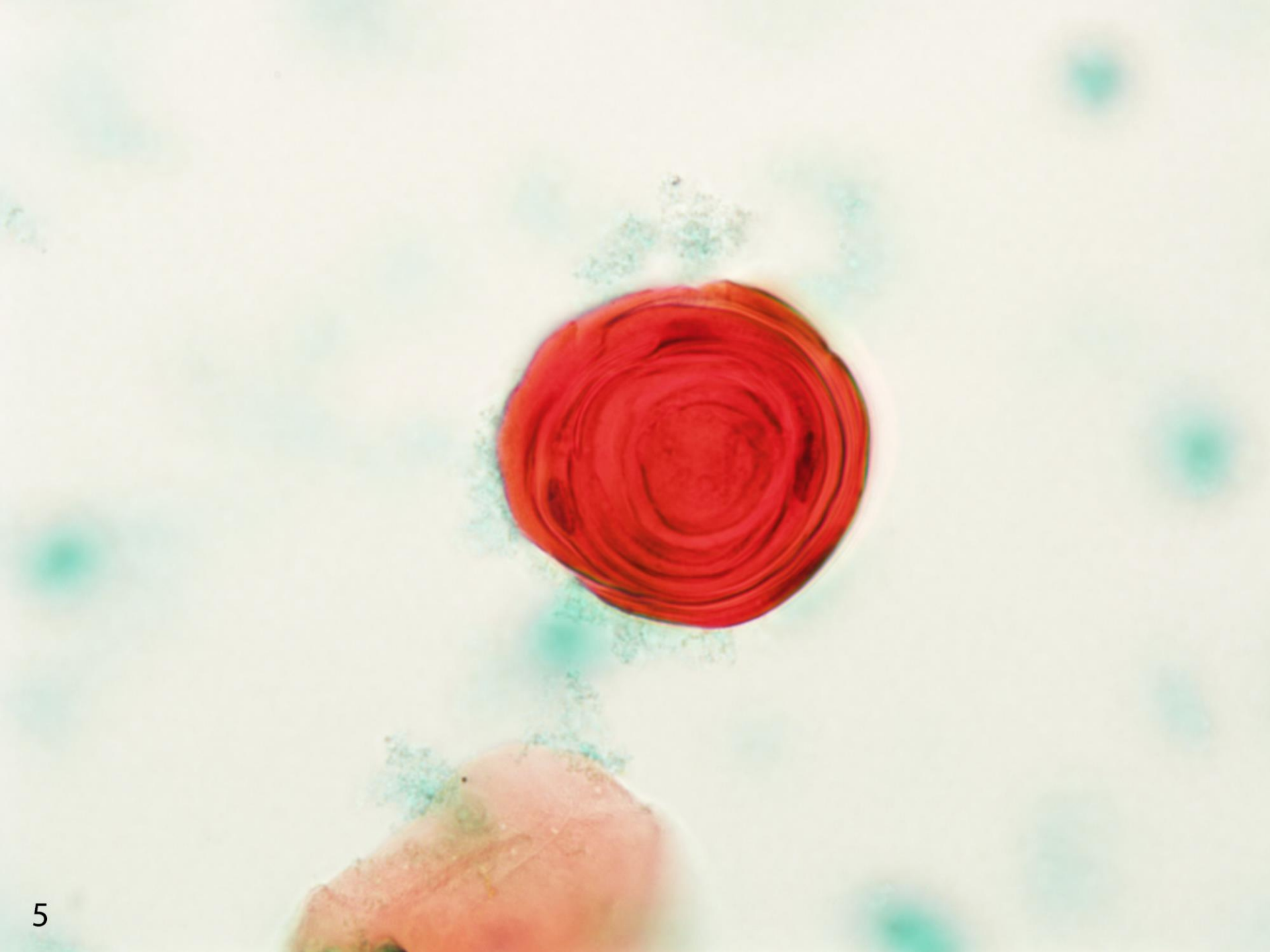

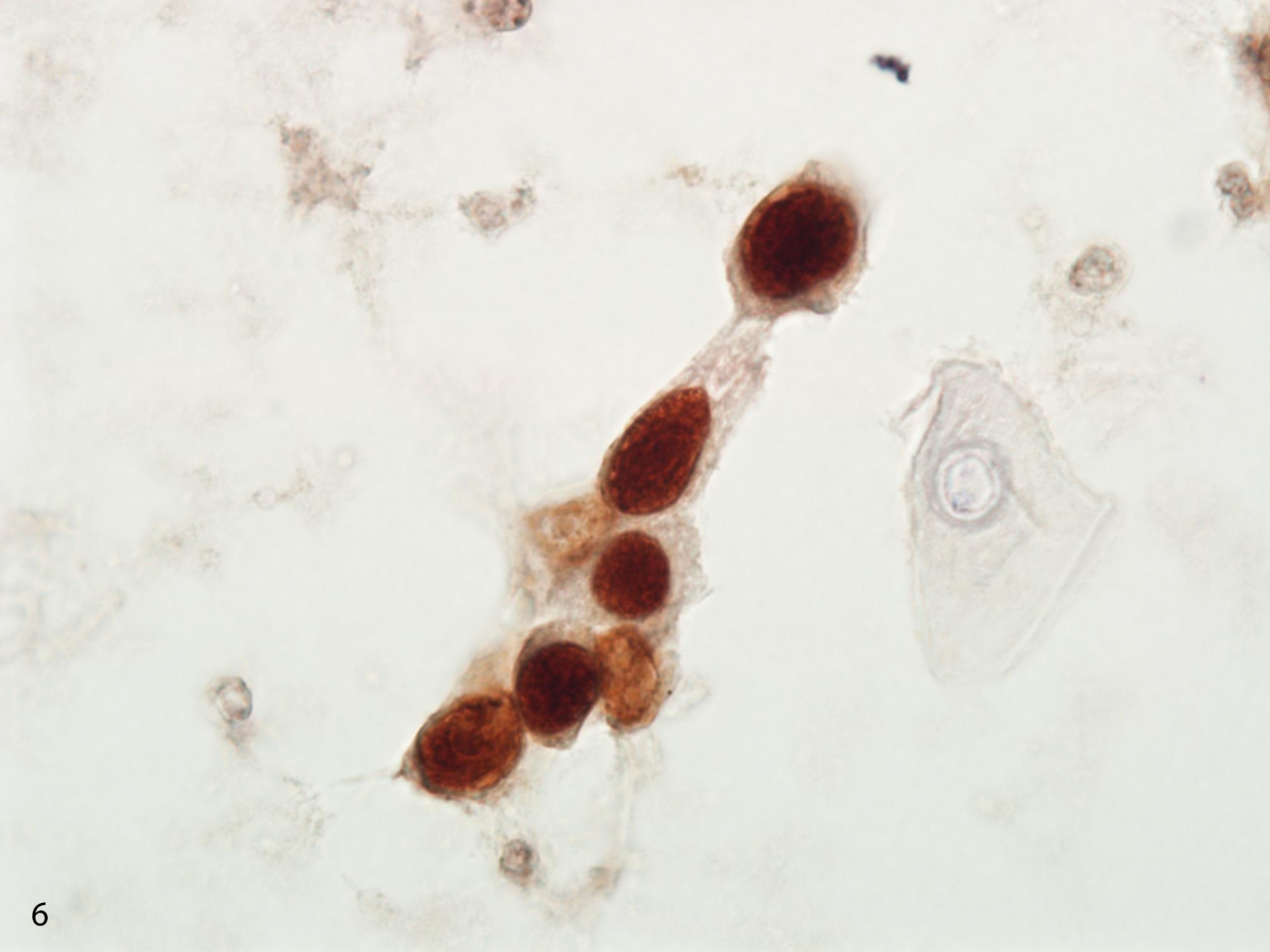

Table 1. Procedure for creating the SurePath slides

| Step | Procedure |
|---|---|
| **1** | Fix the sample with CytoRich$^{TM}$ Red Preservative Fluid* (30 min) |
| **2** | Centrifuge the specimens at 3,000 rpm (2 min) and remove the supernatant |
| **3** | Add distilled water (1.5 mL) to the sediment and resuspend it |
| **4** | Transfer the specimen (0.5 mL) to a settling chamber* (3 times) and mount it on the positively charged slide* (15 min) |
| **5** | Invert the slide rack* and discard the supernatant |
| **6** | Remove the settling chamber and immediately soak in 95% ethanol (30 min) |

*: Becton Dickinson