What has Better Diagnostic Accuracy in Pemphigus? Direct Immunofluorescence of Plucked Hair, Oral Scrapes or Tzanck Smears!

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ABSTRACT Background: Pemphigus is identified by the intercellular deposition of immunoglobulin IgG on direct immunofluorescence (DIF) within skin, hair follicles, and oral mucosa. The objective of this study was to evaluate the diagnostic accuracy of DIF in hair, oral mucosal scrapes, and Tzanck smears, with DIF of skin serving as the reference standard.

Materials and methods: This cross-sectional observational study included patients with clinically suspected pemphigus (n=70) selected through non-probability sampling and compared them with age-matched controls (n=70) who had non-pemphigus bullous lesions. All participants underwent Direct Immunofluorescence (DIF) of hair, oral mucosal scrapes, and Tzanck smears following histopathological examination. The sensitivity, specificity, diagnostic accuracy, and area under the receiver operating characteristic curve (ROC) were calculated.

Results: Of the 70 clinically suspected cases of pemphigus, histopathology confirmed 64 cases (91.4%) as pemphigus vulgaris and 6 cases (8.6%) as pemphigus foliaceus. Perilesional skin biopsy showed a fish net pattern (intercellular IgG deposition) in 100% of pemphigus cases. DIF of the outer root sheath of scalp anagen hair demonstrated this pattern in 94.3% of cases. Tzanck smears from fresh blisters showed pericellular membranous deposits on acantholytic cells in 69.3% of cases. Oral scrapes DIF indicated pericellular deposition around acantholytic cells in 65.7% of cases. Controls (n=4) also exhibited IgG intercellular deposition (fish net pattern) on DIF. ROC curve analysis revealed that hair DIF had a superior diagnostic accuracy (AUC = 0.968), compared to oral scrapes DIF (AUC=0.917) and Tzanck smear DIF (AUC=0.771), respectively.

Conclusion: DIF analysis of hair has superior diagnostic accuracy as compared to oral and tzanck smear.

KEYWORDS: Pemphigus, Direct immunofluorescence, Skin biopsy, anagen hair, Tzanck smears.

INTRODUCTION

Pemphigus is an autoimmune disorder characterized by the presence of autoantibodies against desmoglein Dsg-3 and/or Dsg-1. Pemphigus vulgaris (PV) primarily affects mucosal surfaces and keratin-

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ized skin, often presenting initially with oral lesions. Pemphigus foliaceus (PF) is marked by superficial, flaccid bullae that rupture easily, resulting in shallow erosions with scale crusts. In contrast to PV, PF is associated with antibodies targeting desmoglein 1 (Dsg1) (1,2).

Direct immunofluorescence (DIF) is commonly used for diagnosing pemphigus. Dermatologists often employ both histopathological analysis and DIF to diagnose clinically suspected bullous lesions accurately (3).

In pemphigus, direct immunofluorescence (DIF) demonstrates intercellular IgG deposition within the epidermis. Nonetheless, DIF is invasive, expensive, requires specialized expertise, and necessitates patient consent. Additionally, the characteristic immunofluorescence pattern is also evident in the outer root sheath of hair follicles. Performing DIF in this location, without involving the scalp, yields a sensitivity ranging from 85% to 100% (4).

Direct immunofluorescence (DIF) is a valuable diagnostic tool for pemphigus; however, it is unable to distinguish between pemphigus foliaceus (PF) and pemphigus vulgaris (PV), as both conditions exhibit similar intercellular IgG and complement staining patterns (5-7).

Few studies have compared the diagnostic accuracy of DIF analysis using plucked hair, oral scrapings, and Tzanck smear in confirmed pemphigus cases. This study aims to assess DIF analysis in PV and explore a quick, simple, and cost-effective diagnostic method.

MATERIALS AND METHODS

Study design

A cross-sectional observational study was conducted at a tertiary care institute from June 2018 to June 2021. Approved by the ethics committee, the trial adhered to Helsinki principles and obtained informed consent from all patients. The study followed STARD guidelines.

Diagnosis of Pemphigus

The diagnostic criteria for pemphigus vulgaris (PV) are as follows: (A) presence of blisters or erosions on mucosal areas, with or without accompanying skin lesions; (B) histopathological evidence of supra-basal splitting and acantholysis; (C) Intracellular deposition of IgG and or C3 antibodies on epithelial cell surface in a "chicken-wire" pattern on direct immunofluorescence (DIF)[R].

The diagnostic criteria for pemphigus foliaceus (PF) include: (A) presence of superficial flaccid bullae, erosions, or crusts, typically located on seborrheic areas such as the face, scalp, chest, and back; (B) histopathological evidence of subcorneal acantholysis with cleft formation and scattered acantholytic keratinocytes; (C) intercellular deposits of IgG and or C3 in the epidermis detected by DIF.

The clinical diagnosis was confirmed by a histopathologist. To ensure objectivity, the clinician was not informed of the DIF analysis results.

Study participants.

Inclusion criteria: All newly diagnosed, histopathologically confirmed cases of pemphigus were included before starting therapy, verified through detailed clinical and treatment history at enrolment. A total of 70 participants with pemphigus were selected using non-probability sampling. Age-matched controls (n = 70) with non-pemphigus bullous lesions were recruited from the same population. All subjects underwent medical evaluation in the dermatology day care unit.

Age matching: The age matching was done with the MATLAB software Version 1, which can be accessed using the link https://in.mathworks.com/matlabcentral/fileexchange/66984-age_gender_match-year_gap-group1-group2-. This function randomly ages | matches two groups +/- the year gap given.

Exclusion criteria

Patients who were administered topical or systemic corticosteroids or immunosuppressants prior to inclusion, pregnant or lactating women, and individuals who were uncooperative—defined as those unwilling to provide informed consent or unable to tolerate sample collection procedures (such as mucosal scraping or hair plucking) despite counseling—were excluded from the study.

Sample collection and processing

Under aseptic conditions, a 3-4 mm perilesional skin biopsy was obtained from various sites based on the clinical presentation of each patient. These sites included perilesional skin, oral mucosa, and scalp, depending on the location of the lesions. The biopsies were collected for Direct Immunofluorescence (DIF) and transported to the laboratory in Michel's fluid. The histopathology specimen was fixed in 10% buffered formalin for at least four hours before processing. Tissue sections of 3-4 micrometers were cut on a rotary microtome and stained with hematoxylin and eosin (H&E). Frozen sections from the biopsy were tested by DIF on the same day. If there was a delay, the sample was stored at 4°C and processed promptly thereafter.

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Table 1. Demographic and clinical characteristics

Parameter	Pemphigus	Non-pemphigus	P value
*Age	41.5±15.5	43.9±19.1	0.584
**Gender			
Male	40(57)	35(50)	0.179
Female	30(43)	35(50)	
**Clinical diagnosis			NA
Pemphigus vulgaris	64(91.4)	0	
Pemphigus foliaceous	06(8.6)	0	
Bullous pemphigoid	0	30(21.4)	
Linear IgA disease	0	16(11.4)	
Bullous SLE	0	12(8.5)	
Dermatitis herpetiformis	0	4(2.8)	
Epidermolysis bullosa acquisita	0	2(1.4)	
Vesiculobullous DLE	0	2(1.4)	
Hailey-Hailey disease	0	2(1.4)	
Sub corneal pustular dermatitis	0	2(1.4)	

*Expressed as Mean±SD, ** Expressed as frequency and percentage

For DIF, only anti-human IgG-FITC antibody was used, and the samples were examined under a fluorescent microscope (NIKON ECLIPSE, 495-515 nm)

For DIF on hair, five anagen hairs from the scalp were processed. Only the first 2 inches of the roots were used; the rest was discarded. Hair roots were fixed on glass slides with tape, leaving them exposed.

For DIF on oral scrape smears, samples were obtained by scraping the perilesional area (clinically normal-appearing mucosa next to the erosion) using a blunt spatula.

Tzanck smears were prepared by de-roofing intact fresh blisters. The smears were obtained by scraping the blister roof and floor with the edge of a blunt wooden spatula. The air-dried smears of hair, oral scrapes, and Tzanck were kept unfixed in a moist chamber during transit and stained using Fluorescein Isothiocyanate (FITC) anti-human IgG (Dako), diluted 1:50, for 30 minutes. Following staining, all three smears were rinsed three times in phosphate-buffered saline (PBS) for 5 minutes each, mounted in buffered glycerol, and examined immediately under an immunofluorescence microscope.

For Direct Immunofluorescence (DIF) staining, fresh frozen skin biopsy sections were subjected to three washes with phosphate-buffered saline (PBS) at pH 7.4 for 10 minutes each. Subsequently, the sections were incubated with FITC-conjugated anti-human IgG at a dilution of 1:50. After incubation, the sections underwent an additional triple wash in PBS, followed by mounting in buffered glycerol. The samples were then examined under a fluorescence microscope to identify the characteristic fish-net pattern. If immediate staining was not possible, all smears were stored at –20°C pending further processing.

Sample size calculation

The minimum sample size for a diagnostic accuracy study was calculated using PASS software (Hintze J, 2011). PASS provides tools for various statistical tests and confidence intervals. We estimated the sample size based on disease prevalence and test sensitivity/specificity, ensuring at least 80% power and a p-value below 0.05. The prevalence of pemphigus in India ranges from 0.09% to 1.8% (10). Considering a prevalence of 1.2%, 80% sensitivity, precision error of 1% and aiming for 80% power, with the normal standard variate (Z = 1.96), the estimated sample size was calculated to be 70. The formulae for sample size calculation were [$N=Z^2$ (Sensitivity) (1-sensitivity) / d_2 x P]. In this formulae, N=desired sample size, Z=normal standard variate, P is the prevalence, and d is the precision error.

STATISTICS

Statistical analysis was performed using IBM SPSS Statistics version 29 (IBM Inc, USA). Data normality was assessed with the Shapiro-Wilk test. Descriptive statistics, including mean and standard deviation (SD) for continuous variables, as well as frequencies and percentages for categorical variables, were com-

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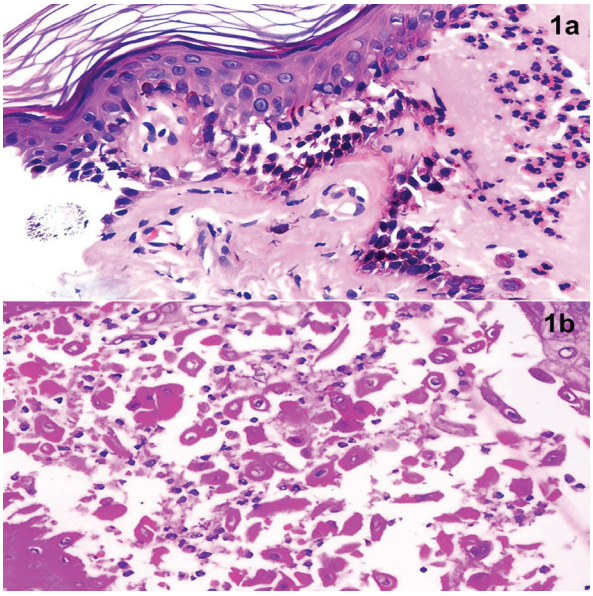

Figure 1a: Photomicrograph of pemphigus showing supra-basal cleft containing acantholytic cells and basal cells (H&E x 100) giving a tombstone appearance.

Figure 1b: Photomicrograph of pemphigus showing numerous acantholytic cells in vesicle (H&E x400).

puted. The independent-samples t-test was utilized to evaluate differences between the means of two independent groups on a continuous dependent variable. The association between two categorical variables was analyzed using Chi-square tests.

Sensitivity and specificity were determined based on a cut-off point on the ROC curve. Cases with a predicted probability of the event (pemphigus) greater than or equal to 0.5 were classified as having the event, while participants with predicted probabilities below 0.5 were classified as not having the event.

All potential cut-off points within the dataset were evaluated, and their impact on the specificity and sensitivity of the test was analyzed. For example, a higher cut-off point increases specificity but reduces sensitivity. This relationship is illustrated by a receiver operating characteristic (ROC) curve, which plots sensitivity against 1 minus specificity. The ROC curve was subsequently constructed, and the area under the ROC curve (AUC) was calculated to provide an overall measure of discrimination. A P value below 0.05 was deemed statistically significant.

RESULTS

Demographic and clinical characteristics

This study compared data from 70 pemphigus patients with 70 age-matched patients who had non-pemphigus bullous lesions based on clinical examination. The recorded information included name, age, gender, and clinical manifestations. Differences in age (P=0.584) and gender (Chi-square test, P=0.179) were not statistically significant (Table 1).

Clinical characteristics of lesions in Pemphigus

Patients with PV presented with lesions primarily on skin and mouth and in patients with PF, typically appear on the face, scalp, and trunk. Multiple-site involvement was observed in 51.4% of patients, while 25.7% had lesions confined to the scalp. All patients with PV had oral and skin lesions but in 20% PV patients, skin lesions were not seen. Genital involvement was noted in 5.7% of cases. In summary, the oral mucosa represented the most frequently affected site in patients diagnosed with pemphigus vulgaris (PV). In contrast, all instances of pemphigus foliaceus (PF) were characterized by lesions involving the skin. The disease duration averaged 34.5±12.4 months, ranging from 2 to 60 months.

Histopathology in patients with pemphigus

Histopathological examination of 70 suspected pemphigus cases revealed supra-basal clefting and acantholytic cells in 64 cases (91.4%), indicating pemphigus vulgaris. In 6 cases (8.6%), acantholysis with splits below the stratum corneum suggested pemphigus foliaceous (Figure 1b). In Pemphigus Vulgaris (PV), basal keratinocytes attached to the basement membrane but exhibiting weak adhesion to each other, creating an appearance akin tombstone aligned in a row (known as the classical row of tombstones) were seen in 58 cases. Additionally, an inflammatory infiltrate, typically comprising lymphocytes, neutrophils, and eosinophils, was observed.

Direct immunofluorescence in patients with pemphigus

Perilesional skin biopsy

Direct immunofluorescence was conducted on perilesional skin biopsies in all clinically diagnosed patients with pemphigus, including those with PV (N=66) and PF (N=6), to confirm the diagnosis. Intercellular deposition of IgG in a fish net pattern was observed in all 66 cases of PV and all 6 cases of PF (100%) (Figure 2a).

Outer root sheath of scalp anagen hair

Scalp lesions were noted in 46 patients, representing 65.7% of the total number of cases, which included both pemphigus vulgaris (PV) and pemphigus foliaceus (PF). However, these lesions were more prevalent among patients with pemphigus vulgaris. Direct immunofluorescence on the outer root sheath

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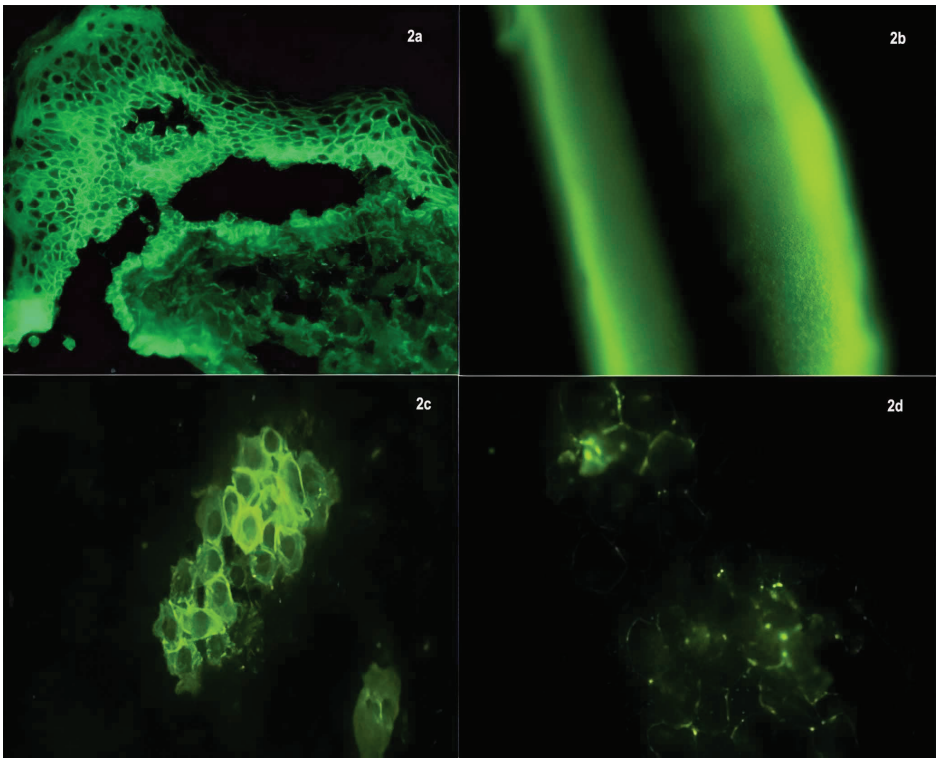

Figure 2a: Photomicrograph of pemphigus showing intercellular deposition of IgG in fish net pattern on skin.

Figure 2b: Photomicrograph of pemphigus showing intercellular deposition of IgG in fish net pattern on ORS of anagen hair.

Figure 2c: Photomicrograph of pemphigus showing pericellular membrane deposits of IgG around individual acanthotic cells in oral scrapes.

Figure 2d: Photomicrograph of pemphigus showing pericellular membrane deposits of IgG around individual acanthotic cells on Tzanck smear.

of scalp anagen hair indicated intracellular deposition of IgG (fish net pattern) in 44 (95.6%) out of 46 cases with scalp lesions (Figure 2b). Additionally, 20 (83.3%) out of 24 patients without scalp lesions also exhibited the classical fish net pattern on DIF. One case with scalp lesions and two cases without scalp lesions showed negative DIF staining on hair DIF. Overall, 66 (94.3%) patients demonstrated a fish net pattern like that found in skin biopsy. Using DIF of the skin biopsy as the gold standard, the sensitivity and specificity for both PV and PF (based on coordinates of the ROC curve, Figure 4b) of ORS of scalp anagen hairs were 75% and 99%, respectively.

Tzanck smears in patients with pemphigus vulgaris.

Out of 70 confirmed cases of pemphigus, fresh blisters were present in 26 (37.1%) patients. Tzanck smears were prepared for these 26 patients, followed by DIF staining. In the DIF examination, pericellular membranous deposits on individual acantholytic cells were observed in 18 (69.3%) out of 26 patients with fresh blisters (Figure 2c). Conversely, 8 (30.7%) patients did not display pericellular membranous deposits on individual acantholytic cells during DIF staining. The DIF on Tzanck smears from fresh blisters showed a sensitivity of 84% and a specificity of 72% in PV patients (Figure 4c).

Oral mucosa scrapings

Oral scrape smears were prepared in all pemphigus cases, both with and without oral lesions. Among the 70 confirmed cases, 40 (57.1%) exhibited lesions in the oral cavity, while 30 (42.9%) had a healthy oral mucosa. Direct immunofluorescence (DIF) examination of the oral scrape smears revealed pericellular deposition around individual acantholytic cells in 46 (65.7%) cases, including four patients without oral lesions. Negative DIF staining was observed in only one patient with oral lesions and in 11 cases without oral lesions (Figure 2d). When comparing DIF of skin biopsy as the gold standard, the sensitivity and specificity of oral scrapes were found to be 82.6% and 91.7%, respectively.

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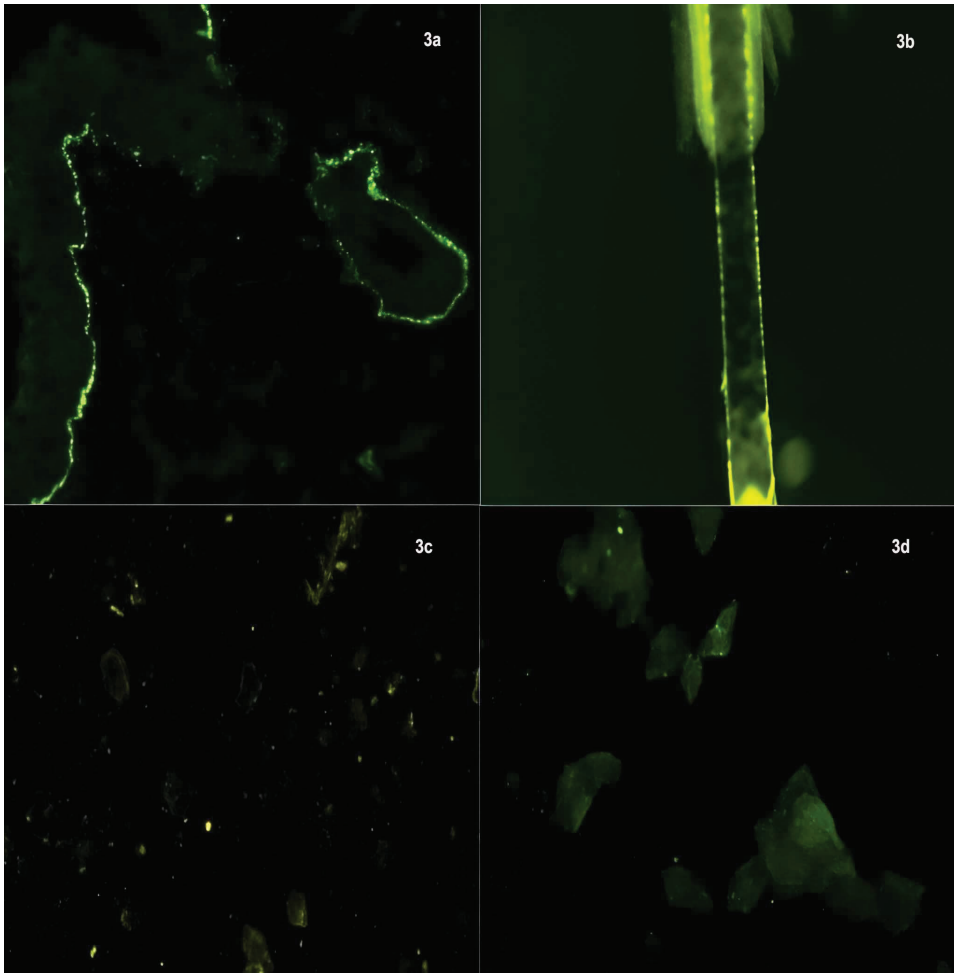

Figure 3a: Photomicrograph showing linear pattern of IgG deposition around BMZ.

Figure 3b: Photomicrograph of non-pemphigus control without intracellular deposition of IgG on ORS of anagen hair.

Figure 3c: Photomicrograph showing absence of pericellular membrane deposits of IgG around epidermal cells.

Figure 3d: Photomicrograph showing absence of pericellular deposits of IgG on oral scrapes.

DIF in controls

All non-pemphigus cases exhibited negative results on DIF analysis of skin, hair, Tzanck smear, and oral scrape specimens (Figures 3a–3d). Nevertheless, two non-pemphigus cases demonstrated DIF positivity around inflammatory and mucosal cells, which may indicate false-positive outcomes. This observation suggests that IgG antibodies are not entirely specific for detecting acantholytic cells. To mitigate such false positives, the application of more specific antibodies targeting inflammatory cells may be advisable.

ROC Curve Analysis: The ROC curve for DIF analysis of anagen hair (Figure 4 a-c) demonstrated excellent diagnostic performance, with an AUC of 0.968 (95% CI, 0.853-1.0), positioning it near the top-left corner of the chart. This indicates superior diagnostic efficacy compared to DIF on oral scrapes (AUC=0.916), followed by DIF in Tzanck smears (AUC=0.771).

DISCUSSION

This study assessed the accuracy of DIF analysis in anagen hair, oral Tzanck smears, and buccal oral scrapes for diagnosing pemphigus, using perilesional skin DIF as the reference standard. Sensitivity results were 75% for anagen hair, 84% for oral Tzanck smears, and 88% for buccal oral scrapes. ROC curve analysis indicated that anagen hair had the highest diagnostic accuracy (AUC=0.968), followed by oral scrapes (AUC=0.917) and oral Tzanck smears (AUC=0.771).

Direct immunofluorescence (DIF) of perilesional skin is the gold standard for diagnosing pemphigus. In this study, DIF revealed intracellular IgG

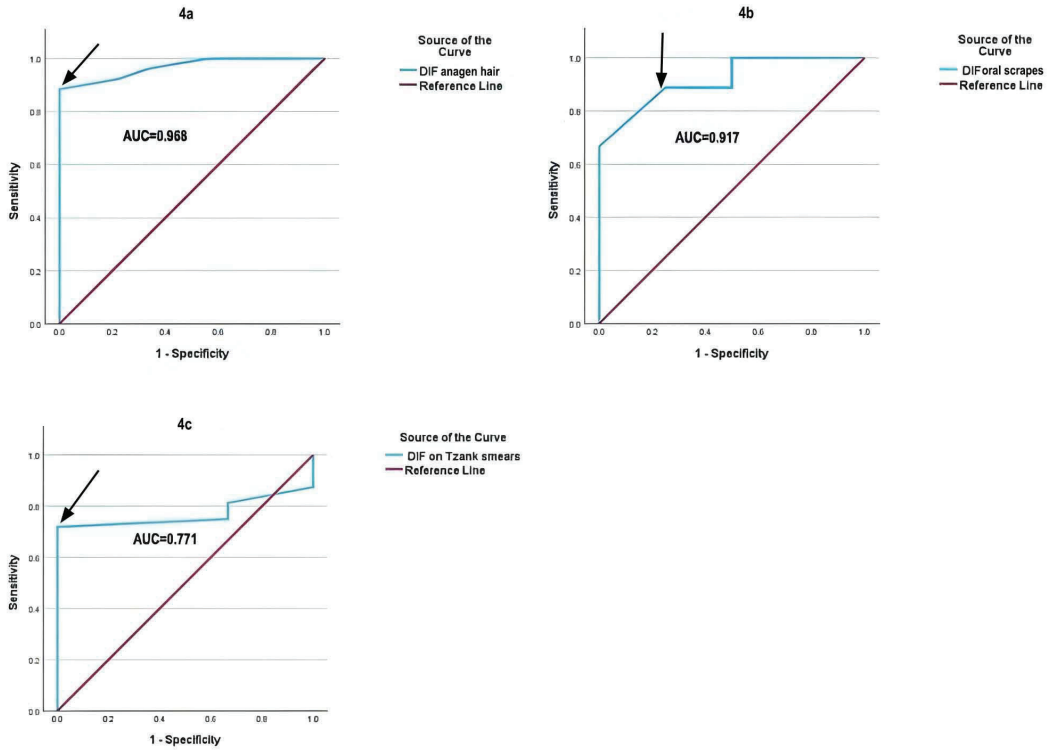

Figure 4 (a-c). Receiver operating curve showing diagnostic performance of DIF anagen hair, oral scrapes, and Tzank smears.

deposition (fish net pattern) in all pemphigus cases (100%), consistent with the reported sensitivity of 90-100% in literature (11-13).

The outer root sheath (ORS) of hair follicles is structurally like epidermal keratinocytes, and autoantibodies can be detected by direct immunofluorescence (DIF) for a longer period compared to perilesional skin (14). Our study demonstrated high sensitivity of anagen hair DIF in diagnosing pemphigus. In a study conducted by Rao *et al.*, intercellular deposition of IgG was observed in the ORS of anagen hair in 85% of patients with pemphigus (n=17) from a cohort of 20 consecutive patients. Another study by Rai *et al.* reported that the sensitivity of hair DIF, in comparison to perilesional skin, was 80% (15). Despite its sensitivity, anagen hair DIF does not differentiate between sub-types of pemphigus.

Tzanck smear examination by staining (cytology) is sensitive but not specific for pemphigus, as other vesiculobullous disorders may also show acantholytic cells (16). Tzanck smear DIF on acantholytic cells, however, relies on fresh blisters and is relatively easier to perform compared to a skin biopsy. In our study, the sensitivity and specificity were 84% and 72%, respectively. A study by Dadras *et al.* assessed the sensitivity and specificity of Direct Immunofluorescence (DIF) on skin/mucosal smears for diagnosing pemphigus in patients with mucocutaneous erosive lesions. The study reported a sensitivity of 82% for oral smears compared to skin biopsies (17). Additionally, He *et al.* found that the sensitivity and specificity of DIF analysis with oral Tzanck smears were 87.80% and 100%, respectively. The higher sensitivity observed in their study may be attributed to a significantly larger number of patients presenting with fresh blisters (18).

In pemphigus, oral lesions often precede skin lesions. Early diagnosis can reduce mortality and morbidity. Cytology of oral scrapes in PV patients typically shows exfoliated acantholytic cells and clusters from adjacent mucosa. Acantholysis with intercellular immunoglobulins on cell membranes confirms pemphigus diagnosis.

A typical acantholytic cell is a large, rounded keratinocyte with a hypertrophic nucleus, hazy or absent nucleoli, and abundant basophilic cytoplasm. Cytoplasmic condensation at the periphery causes a perinuclear halo. Direct immunofluorescence (DIF) positivity on epithelial cells' surfaces can be seen in early stages of pemphigus, even with minimal or no mucosal involvement.

This highlights the potential of DIF to detect subclinical disease and aid in early diagnosis (19). Studies by Laskaris et al, Acosta et al and He W *et al.* demonstrated sensitivity of DIF on oral scrape smears were 100%, 86.0% and 87.8%, respectively (18,20,21). In our study, the sensitivity of oral scrapes DIF was 88%,

which is comparable to the previous study. Additionally, our study showed that oral mucosal scrapes DIF have higher sensitivity compared to anagen hair and Tzanck smears DIF. It can be a straightforward method for diagnosing pemphigus in patients with clinically suspected oral lesions.

A study conducted by Jindal *et al.* evaluated 50 patients with pemphigus and 50 controls using indirect immunofluorescence (IIF) with two substrates: oral mucosa scrapes and normal human skin. The findings indicated that the sensitivity of oral mucosal scrapes on IIF was 80% (22).

This study had several limitations and strengths. Although the number of participants reached the sample size calculated for diagnostic studies (n=75), a larger sample size may be needed to better validate the findings. A small sample size potentially leads to type II error and overestimation. Additionally, fresh blisters were present in 26 out of 70 confirmed cases, allowing Tzanck smears and subsequent DIF staining in only 37.1% of patients. The technique for collecting and processing oral Tzanck smear specimens could influence the results. The prospective study design and the presence of a control group are notable strengths.

Peri-lesional skin biopsy DIF is the gold standard for diagnosing pemphigus. However, DIF analysis of hair shows better diagnostic efficacy than DIF on oral scrapes (AUC=0.968) and Tzanck smears. Since oral lesions often appear before skin lesions in pemphigus, oral scrape DIF can enable early and accurate diagnosis.

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