Microbes and Infectious Diseases 2022; 3(4): 1074-1081

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

## Original article

# The value of PAP and AgNOR techniques in identification of bacterial infections and cytomorphological changes in sputum of Sudanese pango smokers

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**ARTICLE INFO**

*Article history:*
Received 6 January 2022
Received in revised form 23 January 2022
Accepted 4 February 2022

**Keywords:**
Pango
Bacteria
Sudanese smokers
Atypia

**Abbreviations:**
**Ag-NoR:** Argyrophilic Nucleolarorganizer regions
**ASR:** age standardized incidence rate
**CB:** cannabinoid receptor
**CBD:** cannabidiol
**CO:** carbon monoxide
**COPD:** chronic obstructive pulmonary disease
**EA:** Eosin Azure
**HIV:** human immunodeficiency virus
**NOR:** Nucleolar organizer regions
**PAP:** Papanicolaou
**THC:** tetrahydrocannabinol

**ABSTRACT**

**Background:**Sputum cytology" is an example of exfoliative cytology, which is based on shedding of cells derived from the lining of an organ into a cavity from where they can be removed by non-invasive means. **Aim:**The study aimed to assess the value of PAP and AgNOR techniques in identification of bacterial infections and cytomorphological changes in sputum of Sudanese pango smokers.**Methods:**In this study 200 apparently healthy individuals volunteers were selected, of which 100 were pango smoker (cases) and 100 were non-pango users (control), Pap smear and AgNOR was used for the staining of sputum smears. All quality control measures were adopted during specimen collection and processing and diagnosis. Statistical Package for Social sciences (SPSS) software version 16 was used for data entry and analysis.**Results:** Findings demonstrated that the inflammation, indicated by presence of inflammatory cells was detected in 36/88among case group , most of them was chronic type, while only 5/100 had inflammation among non-smokers with significant relation , the *p* value is (0.000).The presence of infectious agent was illustrated among pango smokers most of them was bacterial and Actinomyces with significant relation the *p* value was (0.01). The nuclear atypia was reported among 5 out of 88 of heavy smoker's atypia. While no case of atypia was reported among non-smokers the *p* value is (0.217). **Conclusions:** Pango smoking is a major risk for occurrence of cellular changes that induce the prolifrative activity that increase the risk factor of lung cancer.

## Introduction

Lung cancer is the leading cause of cancer-related deaths in the world among men and woman.Worldwide more than 2 million new cases and almost 1.8 million deaths from lung cancer occurred in 2018 [1]. In South Africa, lung cancer

DOI:10.21608/MID.2022.114978.1228
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similarly ranks as the number 1 cause of cancer deaths [2] with the age standardized incidence rate (ASR) 3.95/100,000 in females and 10.12/100,000 in males.

The most important risk factor for lung cancer remains tobacco smoking. It is estimated that 33.4% of males and 8.3% of females above the age of 15 are consumers of tobacco in South Africa [3]. Other factors such as a family history, poor diet, chronic obstructive pulmonary disease (COPD), ionizing radiation, human immunodeficiency virus (HIV) infection, occupational exposures and air pollution may also predispose to lung cancer [4].

At face value, screening for lung cancer seems highly appropriate, given that smoking is the major identified risk factor (which allows the targeting of high-risk individuals) along with the high prevalence of lung cancer, the high associated morbidity and mortality, the protracted preclinical phase, and the clear evidence that therapy is more effective the earlier the diagnosis is made [5].

The nature of pango came fromcannabis, commonly termed as marijuana, weed, cannabis is the most widely used illicit drug worldwide, only surpassed by alcohol and tobacco when also considering legal substances. Recent investigations have highlighted the therapeutic potential of cannabis, resulting in a resurgence of its consumption for medical purposes. Although cannabis continues to be used mostly for recreational purposes, people increasingly consume it to benefit from its therapeutic properties [6-8].

The primary psychoactive constituent of marijuana is a cannabinoid, delta-9-tetrahydrocannabinol (THC), which produces relaxation, mild euphoria, sedation, and perceptual distortion. There are over 80 other cannabinoids including cannabidiol, cannabinol, and tetrahydrocannabivarin present in marijuana as well as THC.

Delta-9-tetrahydrocannabinol is the principal source of the psychoactive effects associated with cannabis use [8]. These effects result from the activity of THC as a partial agonist of the cannabinoid receptor CB1, which is primarily located in the central nervous system, and CB2, which is predominantly expressed in the peripheral tissues [9]. Delta-9-tetrahydrocannabinolhas observable effects on behavior, nociception, and appetite, as well as anti-inflammatory, antitumor, and antiemetic properties. Tetrahydrocannabinol is also responsible for the psychotropic effects and addictive and reinforcing properties of cannabis [10].

Lung cancer is one of the most important types of cancer that threaten human life; it refers to cancer that develops in any portion that poses the respiratory tract. The remarkable cause of lung cancer is smoking with all types.

The study aimed to assess the value of PAP and AgNOR techniques in identification of bacterial infections and cytomorphological changes in sputum of Sudanese pango smokers.

## Materials and Methods

This was a descriptive cross sectional study aimed to evaluate the sputum cytological patterns among heavy smokers (pango) individuals. Samples were collected from different areas in Khartoum state. The study was carried out during the period from December 2020 to November 2021.

### Study population

Sudanese pango smoking users as test group and non pango smokers as control group.

### Sample size

Hundred samples were collected from pango smokers as test group and 100 samples from non pango users as control group, and then two slides were made from each respondent and stained by PAP and AgNOR stains .

### Specimen

Sputum specimen was taken from any participant. A questionnaire to obtain essential data filled with every respondent.

### Papanicolaou staining technique

Smears were fixed with 95% ethanol for 15 minute then rinsed in tap water, then added harris Hematoxylin 1-3 minutes then rinsed in tap water, then dipped in 95% ethanol, then add eosin azure 2.5 minutes, then dipped in 95%ethanol 2 changes, then 100%ethanol for 1minute, clear in 2 changes of xylene 2 minutes for each, then mounting with DPX [11].

### Argyrophilic Nuclear Organizer Regions (AgNOR) staining method

The air dried smears were stained according to the AgNOR staining method. Working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All smears incubated with this silver solution for 30 minutes at room temperature in a dark area and they were protected in the dark until each slide analyzed.

Two cytopathologists examined and interpretated the silver-stained cells under light microscope (Olympus BX-51, Japan) at 10x & 40x magnification. All smears screened horizontally from left to right and AgNORs counted in the nuclei of the first 50 non-overlapping, inner layers, nucleated epithelial cells. Superficial cells with pyknotic nuclei not counted. The AgNOR count made adopting the method described by **Crocker et al.**[12]. Argyrophilic Nuclear Organizer Regions, which are visible as black-dark brown dots located within the nuclei of the cells, was counted; overlapped black dots counted as one structure [11].

**Statistical analysis**

The mean and standard deviation (mAgNOR ± SD) of AgNOR dots in 100 tumor nuclei and Proliferative Index (pAgNOR) i.e. the percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei were estimated. A similar thing was done for the 50 tumor nuclei. The data collected were statistically analyzed to generate Pearson correlation coefficient (*p*-value) using Statistical Package for Social Sciences (IBM SPSS Statistics 21) to compare the values observed in each tumor grade and the values observed for counting 50 and 100 tumor nuclei [13].

Chi square test was applied for the comparison of numerical and categorical data. Then *p*-values added indicating statistical significant and highly significant difference. (e.g.*p* values <0.05considered statistically significant).

**Ethical consideration**

All participants were fully informed about the aims and outcomes of the study, and were asked to sign a written consent before taking the specimen by the pathologist in-charge. The results have been shown to and discussed with the patients. Ethical approval was obtained from the National University Ethical Research Committee in accordance with the Declaration of Helsinki Principles, and the agreement was taken from all patients before sample and data collection. The patient's information were highly secured and not used for other purposes than scientific inquiry. Risk and benefits for the patients from outcomes of the research insured.

**Approval reference number**: NU-REC/01-020./09

**Approval date**: 2/11/2020

**Results**

A total of 200 samples of sputum were included in this study, all of them were Sudanese participants smokers and non smokers. The age of the study population ranged from 20 to 30 years , with mean age of 23.38 [SD=1.88] years with most frequent age 23 years. Duration of smoking ranged from 1 to 10 years with mean of 3.46 years.

**Number of cigarettes per day:**

Number of smoking cigarettes per day from 1 to 8 cigarettes per day .

**Infections:**

Out of 88 samples 10 samples had a bacterial infection, 13samples had Actinomyces infection, and one sample bi-infection and 64 samples were negative, with statistically significant difference when compared with control group, the *p* value was **(0.003)**.

**Inflammations:**

Out of 88 samples 10 sample had acute inflammation, 26 samples had chronic inflammation and 52 samples were negative , with statistically significant difference when compared with control group, the *p* value was (**0.002**).

**Inflammatory changes:**

Out of 88 samples36 samples had inflammations among smokers, while only five cases were reported among non smokers, the *p* value was highly significant (**0.001**).

**Cellular atypia:**

Out of 88 samples 5 samples had nuclear atypia, and 73 samples were negative, with statistically non significant difference when compared with control group, the *p* value was (0.217).

**Table 1.** Comparison of AgNOR results between case and control groups.

| AgNOR | Mean | Std.deviation | *p*.value |
|---|---|---|---|
| **Case** | 4.510 | 1.880 | **0.001** |
| **Control** | 2.220 | 0.720 | |

Chi square test was applied for comparison between case and control groups, *p* value = 0.001, *p* value <0.05 which showed statistically significant).

**Table 2.**Frequency of inflammation.

| Variables | Inflammation | | Total | *p* value |
|---|---|---|---|---|
| | Yes | Without inflammation | | |
| **Case** | 36 | 52 | 88 | **0.001** |
| **Control** | 5 | 95 | 100 | |
| **Total** | 41 | 147 | 188 | |

Chi square test was applied for comparison between case and control groups based on presence of inflammation, *p* value = 0.001, *p* value <0.05 which showed statistically significant).

**Table 3.**Relation between case and control with type of inflammation.

| Variables | | | Total | *p*value |
|---|---|---|---|---|
| | Acute | Chronic | | |
| **Case** | 10 | 26 | 36 | **0.002** |
| **Control** | 3 | 2 | 100 | |
| **Total** | 13 | 28 | 136 | |

Chi square test was applied for comparison between case and control groups based on type of inflammation, *p* value = 0.002, *p* value <0.05 which showed statistically significant).

**Table 4.**Relation between case and control with type of infection.

| Variables | Infection | | Total | *p*value |
|---|---|---|---|---|
| | Yes | No infection | | |
| **Case** | 24 | 64 | 88 | **0.002** |
| **Control** | 6 | 94 | 100 | |
| **Total** | 30 | 158 | 188 | |

Chi square test was applied for comparison between case and control groups based on type of infection, *p* value = 0.002, *p* value <0.05 which showed statistically significant).

**Table 5.** Distribution of study population according to type of infection.

| Variables | | | | Total | *p* value |
|---|---|---|---|---|---|
| | Actinomyces | Bacterial | Bi infection | | |
| **Case** | 13 | 10 | 1 | 24 | **0.003** |
| **Control** | 6 | 0 | 0 | 6 | |
| **Total** | 19 | 10 | 1 | 30 | |

Chi square test was applied for comparison between case and control groups according to type of infection, p value = 0.003, p value <0.05 which showed statistically significant).

**Table 6.** Frequency of atypia.

| Variables | Atypia | | Total | *p* value |
|---|---|---|---|---|
| | Yes | Normal cell | | |
| **Case** | 5 | 83 | 88 | **0.217** |
| **Control** | 0 | 100 | 100 | |
| **Total** | 5 | 183 | 188 | |

Chi square test was applied for comparison between case and control groups based on frequency of atypia, *p* value = 0.217, *p* value >0.05 which showed statistically insignificant.

## Discussion

Cannabis is the most abused drug in most countries of the world [14]. An estimated 147 million people use cannabis globally, mainly for recreational purposes [15]. In Canada, 4.2 million people aged 15 and older reported using cannabis products in the previous 3 months, and a third of surveyed youths had consumed cannabis at least once by their 15$^{th}$ birthday [16].

Smoking cannabis has adverse effects on multiple human organs including the oral cavity [17]. The plant comprises over 400 chemical entities with the two main compounds,THC and cannabidiol (CBD), shown to have opposing effects on many human organs. People who smoke cannabis usually experience an altered mental state (psychoactive feeling) that commences within a few minutes and can last up to 3 hours [18]. The long-term effects of cannabis use include low birth weight, structural, functional and chemical changes in the brain, early onset psychosis, strokes, testicular cancer, suicide tendencies and deficiency in motor function and learning [17]. It is estimated that up to 17% of people who start consuming cannabis as adolescents will develop cannabis use syndrome (irritability, anger, depression, difficulty sleeping, craving and decreased appetite) [19]. In the oral cavity, smoking cannabis has been associated with periodontal disease [20], dental caries [21] and oral cancers [22].

The history of substance misuse in Sudan, especially of locally produced alcohol and drugs, can be traced back for many centuries. For several decades, locally made alcohol beverages and locally cultivated cannabis have been the two main substances of use among certain groups in Sudan [23].

Over the past decade, the drug scene in Sudan has shown a rapid surge of use of many substances, including misuse of prescription drugs. Commonly misused prescription medicines among young people include tramadol (also known as strawberry or pink); benzodiazepines, e.g. clonazepam (Roche); cough syrups and antihistamines. Other substances include trihexyphenidyl (also known as kharsha), anticonvulsants and neuropathic pain agents (pregabalin and gabapentin), and antipsychotic medications (e.g. quetiapine). Owing to the absence of stringent prescription and dispensing monitoring systems, most of these medicines can be obtained without prescriptions [24].

In this study the significant presence of cytological atypia and metaplasia among pango smokers compared to control , considered as strong evidence that pango smoking is one cause of lung epithelial proliferative activity abnormalities, which may develop to lung pre-cancer or cancer lesion, the use of smoking was previously reported to induce cytological atypia and premalignant change of lung.

Similar results were reported in other studies such as **Hubers et al.**[25],this means that consecutive annual sputum examination increases the chance of detection of sputum atypia and thus increases the chance of detection of lung cancer. The case group has significantly higher mean AgNOR count than control group4.510 (±2.1), and this indicates that pango use increases cellular proliferative activity. Furthermore, mean nuclear area of the smoker group is significantly higher than the nonsmokers ($p< 0.01$), and mean nuclear area of the smoker group is significantly higher than the nontobacco users, which is a similar finding to another study reported elsewhere. In regard to the infection and inflammatory conditions, cases were more susceptible than controls, and this was found to be statistically highly significant ($p< 0.0001$). Similar findings obtained by **El Mahi** in 2018 [26].

The nicotine and exposure of respiratory tract epithelium to the cigar irritating substances (heavy metals such as arsenic, cobalt, chromium,

and lead) and carbon monoxide (CO) are major causative factors. Smoking was associated with a 35- to 50 percent increase in the risk of respiratory tract infections and inflammation [27]. These findings are in agreement with other studies suggesting that use of tobacco disturbs the normal maturation of the epithelial cells.

The increase of cytological atypia among older people due to tobacco exposure was previously reported by a study from Sudan [28]

The mortality from SARS-COV-2 infection is higher among patients with cancer than in the general population. In a cohort study of 928 cancer patients confirmed with COVID-19 infection in the US, Canada, and Spain, the all-cause mortality rate was high at 13% [29].

Cytology samples obtained from exfoliative sources procedure can be used to detect microorganisms and/or the associated cytopathologic effects caused by an infection. There are many advantages to utilizing cytology samples as an adjunct to routine microbiology laboratory methods. For example, cytology samples can be obtained by non-invasive and minimally invasive techniques, and interpretation is affordable, accurate, and fast [30].

Routine cytology stains, including the PAP stains, can adequately identify a number of microorganisms. In general, the PAP stain provides a high-quality view of nuclear and cytoplasmic detail, making it an ideal stain for assessing a host's response to an infectious agent, such as viruses and bacteria [30].

Nucleolar organizer regions (NORs) are loops of DNA that have encoding for rRNA and play an important role in protein synthesis in cells. They chemically bind with silver, the complex formed is referred to as argyrophilic NOR (AgNOR) which is observed to count their numbers [31].

Nucleolar organizer regions count is a simple, cost-effective, and reliable method that can give a quantitative measurement for the risk of lung neoplastic transformation. For at risk-population (tobacco users), it is recommended to perform the AgNORs method beside sputum cytology [32].

**Gulati et al.** reported that the AgNOR technique is simple, inexpensive and a useful adjunct to routine histopathology, to evaluate pulmonary lesions. The counts, however, cannot be standardized for a particular lesion as there are inter-laboratory variations [33].

The findings of **Turan Sönmez and Eröz** revealed that AgNOR protein levels were elevated during a chronic obstructive pulmonary disease exacerbation compared with healthy control subjects and there was a positive correlation between $pCO_2$ levels and mean AgNOR number [34].

## Conclusions

Smoking induces cellular proliferative activity leading to an increased risk of lung cancer. Sputum cytology might be helpful to identify high risk individuals who could benefit from more diagnostic examination and/or be enrolled into lung cancer chemoprevention trials.

## Competing interests

The authors declare that they have no competing interests.

**Funding:** None.

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