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Presence and metabolic activity of infectious agents as important clinical factor in gynecological cytology

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Abstract: Gynecological cytology, which involves microsopic observation of a cervical sample, is the gold standard in screening of cervical cancer even todayand commonly known as the Pap smear. Regular screening for human papilloma virus (HPV) in postitive Pap smears improved understanding of relationship between HPV and cervical cancer, however, not much attention is given to the other infectious agents such as Gardnerella vaginalis, Trichomonas vaginalis, yeasts and mixed vaginal bacterial flora. In this study, we investigated the relationship between these infectious agents and morphological changes of the cervical cells as well as amount of released microbial metabolic products and their possible role in the development of cervical cancer.
Keywords: Aspartyl proteinase, cervical cancer, infectious agents, Pap smear

1. INTRODUCTION

Gynecological cytology is a field of medical pathology that is based on detection of cervical carcinoma and its precursors. The most common procedure used in gynecological cytology is the Pap smear[1]. This test identifies risk or the presence of cervical cancer and was introduced by George Nicolas Papanicolau who has been regarded as the father of modern cytopathology ever since[2]. Pap smear became a gold standard in gynecological cytology and remains the most successful cancer-screening test ever developed. Cervical precancerous lesions are associated with abnormalities in cytologic preparations that can be detected long before any abnormality is visible on gross inspection. Those lesions are present in various degrees, from reversible metaplastic to dysplastic and in the end, neoplastic irreversible changes on cervical epithelium [3-5].Lesions are usually associated with prolonged impact of different kinds of noxious agents. One of the most known noxious agent is the Human papilloma virus (HPV) which is considered as the most oncogenic infectious agent associated with cervical carcinoma. In addition to HPV, there are numerous infectious agents which have harmful effects on the physiological functions and histological architecture of the female reproductive system. Gynecological infections are one of the most abundant diagnoses seen in modern gynecological practice and their understanding has the crucial part in pathogenesis and treatment. The great importance in combat with infections has the anatomy of the female reproductive system itself, in particular commensal vaginal microbiota. Vaginal commensal microbiota consists of predominantly aerobic with approximately six different species of bacteria. Presence of the commensal flora and associated metabolic byproducts of microorganisms have a key role in defense against infectious agents by preventing their overgrowth. The most abundant genus in vagina is *Lactobacillus* which maintains normal vaginal pH through production of lactic acid. It also produces hydrogen peroxide which is toxic to catalase negative bacteria[6-8].Disturbance in commensal vaginal microbiota results in increased vaginal discharge and infections manifested as colpitis (vaginitis) and cervicitis. Vaginal discharge is the pathognomonic sign of the infection and based on the causative agent can be divided into the six broad categories. Prolonged presence and unrecognized infection can lead to the irreversible changes on vaginal and cervical epithelium. Each microorganism possess certain abilities called virulence factors which enhance their effects in pathogenesis of disease and resistance to therapeutical methods or substances. Those abillities are known as virulence factors. In this study we investigated the biofilm formation, metabolic activity and concentration of aspartyl proteinase in microorganims isolated from the cervical and vaginal samples including mixed bacterial vaginal flora, *Gardnerella vaginalis*, *Trichomonas vaginalis* and *Candida spp*.[9-12].

2. MATERIALS AND METHODS

For this study, 55 patients from the gynecological polyclinic “Korak do života” in Tuzla, Bosnia and Herzegovina signed an informed consent and provided the important clinical data through the conducted survey, in period October 2020-January 2021. Data included: patient’s age, year and results of the last Pap smear, presence of comorbidities in terms of acute or chronic diseases and susceptibility to urinary infections. Cervical and vaginal smears were collected from the each patient. Samples were stained by conventional Papanicolau

staining method and Pap smear results were based on microscopy. In addition, cervical and vaginal smears were inoculated into growth media and the resulting isolates were tested for biofilm formation.Finally, the isolates were assayed for total protein concentration and aspartyl proteinase concentration.

2.1. Strains and growth conditions

Microorganisms from the cervical and vaginal samples collected from the 55 patients included in the study were isolated prior to this study by conventional microbiological identification and isolation methods. Microorganims included bacteria belonging to mixed vaginal flora, *Gardnerella vaginalis*, *Trichomonas vaginalis* and *Candida spp*.. Isolated microorganisms were tested for biofilm forming capacity. Prior to testing, bacterial isolates were inoculated in 3 ml of trypticase soy broth media supplemented with 1% of glucose and were incubated under aerobic conditions for 24h at 37°C.

2.2. Sample preparation

After 24h incubation in growth media, 100 µl of bacterial culture was taken and mixed with 400 µl of 1% bovine serum albumin (BSA). Prior the procedure, 1% BSA and 10% trifluoroacetic acid were diluted with 0.1 M citrate buffer with pH-value 3.5. Prepared samples were incubated again at 37°C for 30 minutes. The reaction was stopped by adding 1 ml of 10% trifluoroacetic acid. After this, the samples were centrifuged for 10 minutes at 1000 rpm. After centrifugation step, the supernatant was obtained and isolated. From the isolated supernatant, total protein concentration and aspartyl proteinase concentration were determined, as described below.

2.3. Protein assays

Total protein concetrations and aspartyl proteinase concentrations were determined from the obtained supernatants. Protein assays were conducted by the Thermo Scientific Multiscan FC spectrophotometer. Protein assays measured absorbance of the test samples at 260 nm and 280 nm according to the Warburg Christian method (78). For determination of total protein concentration, one measurement for each sample was performed. For determination of aspartyl proteinase concentration, measurements were performed for each sample in triplicates and the total protein concentration was calculated following the Warburg Christian equation: mg of protein/ml = $1.55 \times A_{280} - 0.76 \times A_{260}$.

2.4. Testing for biofilm forming capacity

Test tubes with bacterial cultures were incubated for 24 h at 37°C. After incubation, 2 ml of the cultures were frozen while the rest of the cultures from the test tubes were decanted and washed with phosphate buffered saline (PBS) with pH 7.5. After that, the tubes were turned upside down and allowed to dry for 20 minutes. When tubes became dry, 1 ml of 0.1% crystal violet stain was added. Important step was to ensure that crystal violet comes in contact with all sides of the test tube and cells attached on the plastic walls of the test tubes. After incubation of 5 minutes, the crystal violet stain was washed with double-distilled H_2O and test tubes were observed for biofilm formation. The test tubes with visible violet film were considered positive for biofilm formation. The amount of biofilm forming capacity was established based on intensity and visibility of the biofilm as well as the number of the cells. Based on this criteria, biofilms formed were categorized as weak, moderate and strong showed in Fig. 1.

Fig. 1. Biofilm forming capacity graded as (right to left): (-) negative, (+) - weak, (++) - moderate and (+++) - strong.

2.5. Statistical analysis

We obtained a data reports by Thermo Scientific Multiscan FC spectrophotometer. Data included obtained values of total protein concentration and aspartyl proteinase concentration from the cervical and vaginal samples taken from the 55 patients. Microsoft Excel was used for data import and statistical analysis. Statistical analysis included an Independent Samples t-Test between the two groups. More precisely, values from the vaginal samples represented one group and values from the cervical samples represented another group. A *p*-value less than 0.05 was considered statistically significant.

3. RESULTS

The aims of this study were to investigate biofilm forming capacity of the infectious agents isolated from the cervical and vaginal smears, to establish how metabolic activitiy of infectious agents isolated from cervical and vaginal smears changes in correlation with their concentration and to investigate putative correlation of secreted aspartyl proteinase by isolated infectious agents and changes in cervical histology and morphology.

3.1. Diagnosis prevalence

Analysis of the data obtained from the 55 patients included in the study showed the most common diagnoses estimated by the gynecological examination (Fig. 2.). The most prevalent diagnosis was the colpitis, either isolated (65%) or associated with cervicitis (16%). The remaining diagnoses included menopause (11%), cervical intraepithelial lesion or CIN (4%), isolated cervicitis (2%) and colpitis with HPV infection (2%).

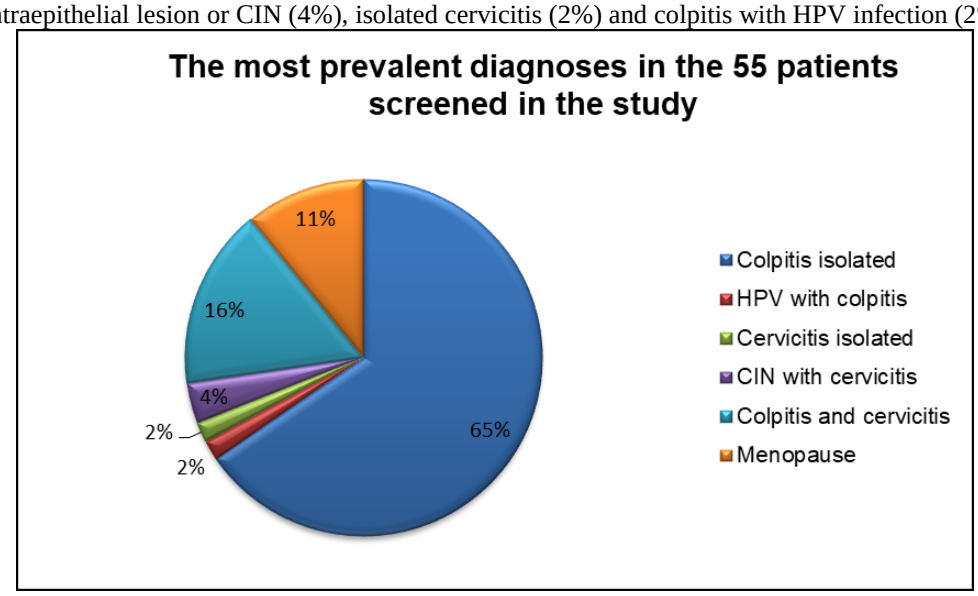

Fig. 2. The most prevalent diagnoses estimated by gynecological examination in the 55 patients included in the study.

3.2. Abnormal vaginal discharge prevalence in correlation with causative agent

Further analysis showed the most prevalent infectious microorganisms as causative agents of the abnormal vaginal discharge (Fig. 3.). Microorganisms were detected by conventional microbiological identification and microscopy. Interpretation of the data showed that the most prevalent microorganisms which caused abnormal vaginal discharge included: mixed bacterial vaginal flora (35%) considered as group III of the vaginal discharge, parasite *Trichomonas vaginalis* (28%) considered as the group V of the vaginal discharge, bacteria *Gardnerella vaginalis* (22%) and yeast *Candida albicans* (15%) considered as group VI of the vaginal discharge.

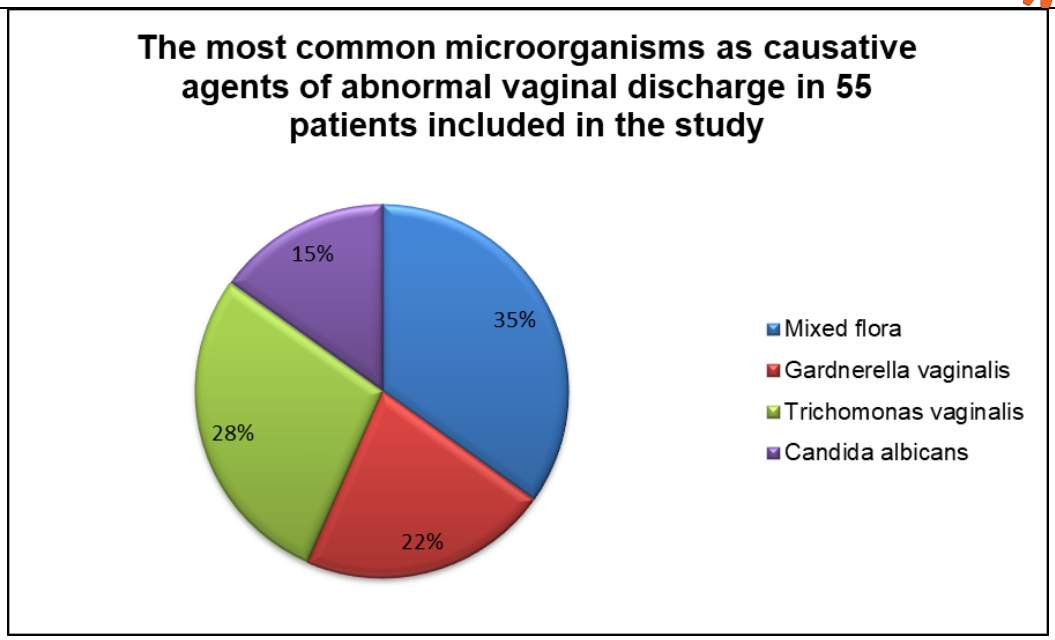

Fig. 3. The most common microorganisms as a causative agents of abnormal vaginal discharge in 55 patients included in the study.

3.3. Biofilm forming capacity

We tested microorganisms from the vaginal and cervical samples taken from the 55 patients included in the study for biofilm forming capacity Results showed that out of 55 tested vaginal samples, 32 (58.1%) were capable for biofilm formation. Out of the 32 vaginal samples positive for biofilm formation: 25 (78.1%) samples had weak (+) biofilm forming capacity and 7 (21,8%) samples had moderate (++) biofilm forming capacity. Out of the 55 vaginal samples, 23 (41.8%) were negative for biofilm formation.Results further showed that out of the 55 tested cervical samples, 28 (50.9%) were capable for biofilm formation. Out of the 28 cervical samples: 20 (71.4%) samples had weak (+) biofilm forming capacity, 7 (25%) samples had moderate (++) biofilm forming capacity and 1 (3.5) sample had strong (+++) biofilm forming capacity. Out of the 55 cervical samples, 27 (49%) of the samples were negative for biofilm formation. Out of the 46 patients whose vaginal and cervical flora had shown capability of forming biofilm, 39 (84.7%) of them had present mixed bacterial flora, 31 (67.49%) of them along with mixed bacterial flora were positive on *Gardnerella vaginalis*, 27 (58.7%) of them along with mixed bacterial flora had associated infection with parasite *Trichomonas vaginalis* and 15 (32.6%) of them along with mixed bacterial flora were positive on *Candida spp*.. It is important to notice that in 3 patients, where microorganisms formed strong biofilm (++ and +++), we had observed the presence of mixed bacterial flora. In 8 patients, we observed mixed bacterial flora along with *Gardnerella vaginalis* in high concentration and in 5 patients along with mixed bacterial flora, *Gardnerella vaginalis*, parasite *Trichomonas vaginalis* was also detected. According to the biofilm forming capacity, we can not exclude a possible synergism between those microorganisms.

3.4. Microbial metabolic activity

We investigated the microbial metabolic activity from the vaginal and cervical samples. Total protein concentration was used as a measure of microbial metabolic activity. Results showed that protein concentration was significantly higher in cervical samples than in vaginal samples in 39 (71%) of the patients. The sample numbers 4, 24, 26, 29, 30, 36, 47, 54 especially showed the higher significant difference between vaginal and cervical samples presented in the Fig.4.

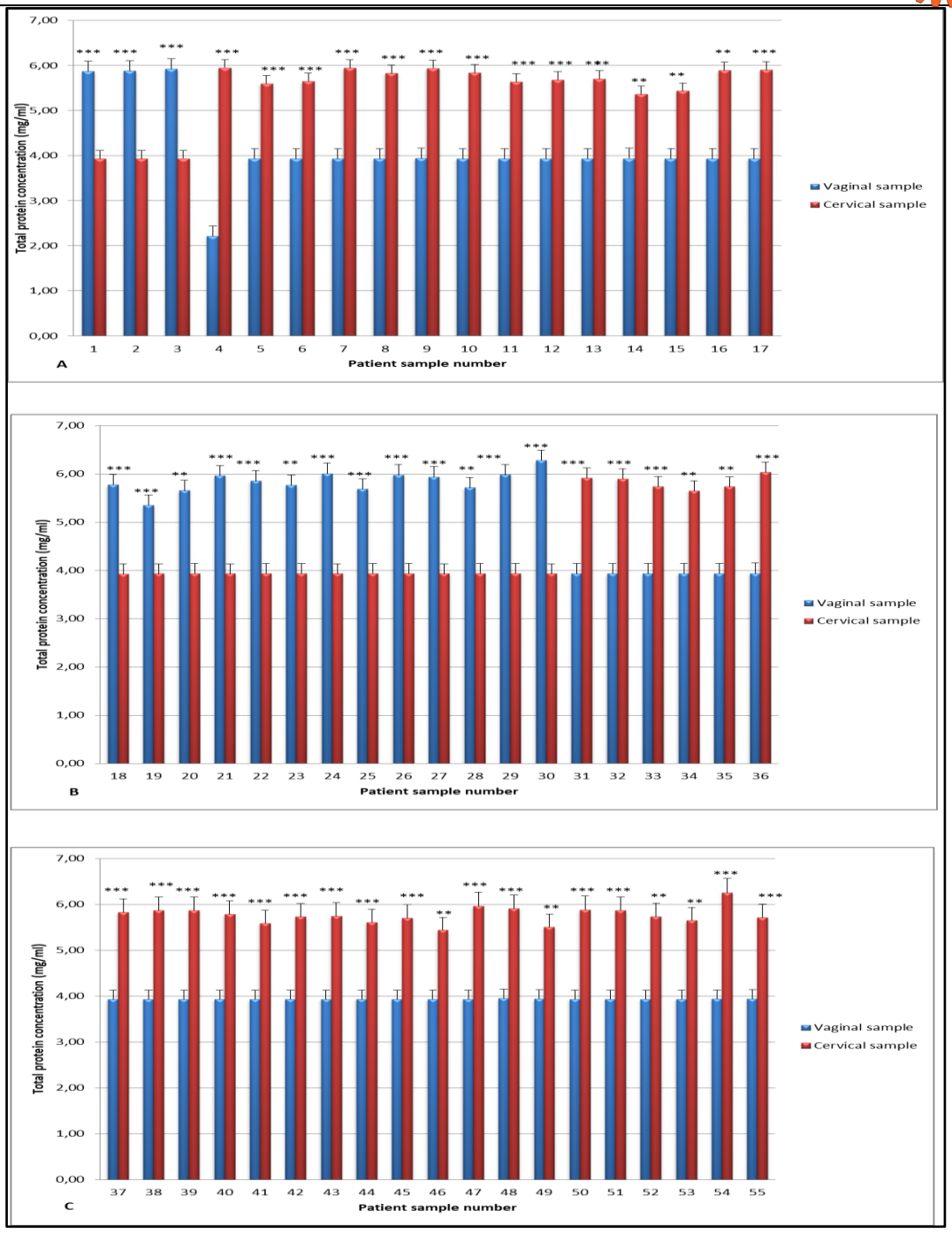

Fig.4.A-C Total protein concentration in 55 vaginal and cervical samples.*(*t-Test independent samples, ****p<0.001, **p<0.01, *p<0.05)*

3.5. Concentration of aspartyl proteinase

From the vaginal and cervical samples, we also determined the concentration of an enzyme aspartyl proteinase. Results showed that concentration of aspartyl proteinase was significantly higher in cervical samples than in vaginal samples in 27 (49.09%) of the patients. The sample numbers 12, 23, 29, 38, 41, 45 and 46 especially showed the higher significant difference between vaginal and cervical samples presented in the Fig. 5.

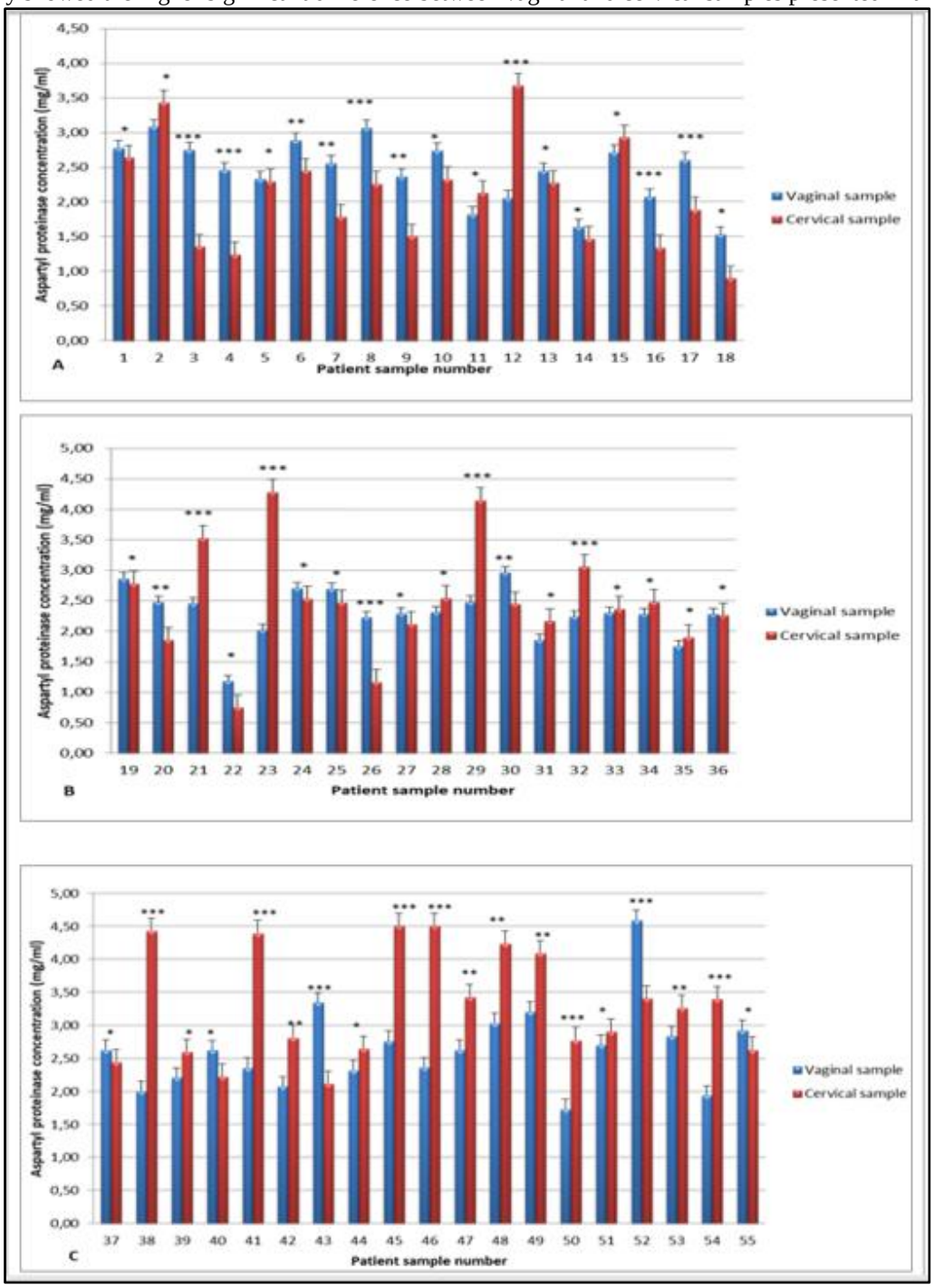

Fig. 5.A-C Concentration of aspartyl proteinase in 55 vaginal and cervical samples. *(*t-Test independent samples, ****p<0.001, **p<0.01, *p<0.05).*

3.6. Low and high squamous intraepithelial lesions

Out of 55 patients, 2 patients were positive for intraepithelial lesion (3.63%), respectively one patient for LSIL (patient 47) and one patient for HSIL (patient 46). In cervical samples in both of these patients, elevated microbiological excretion of aspartyl proteinase was found in concentrations of 4.51 mg/ml for patient with

HSIL and 3.43 mg/ml for patient with LSIL. Formation of biofilm by microbes isolated from the cervical sample was observed in one patient with LSIL, while microorganisms isolated from the vaginal smear did not form biofilm in neither patient. In both of these patients, associated infection with mixed flora, *Gardnerella vaginalis* and *Trichomonas vaginalis* was present.

4. CONCLUSIONS

Data obtained from the protein assays of vaginal and cervical samples taken from the 55 patients included in the study, showed total protein concentrations which was used as a measure of metabolic activity of the infectious agents. Our results showed that total protein concetration was significantly increased in 16 (29%) tested vaginal samples. The 39 (71%) of the patients had higher levels of total protein concentration in cervical samples. It was possible to see, that aspartyl proteinase concentration was also significantly higher in 27 (49.09%) of cervical samples from the total number of 55 cervical samples. Out of them, 21 (77.78%) of the patients had bacterial flora capable for formation of the biofilm.From the total number of 55 vaginal samples, aspartyl proteinase concentration was increased in 17 (30.9%). The 12 (70.6%) of those samples formed biofilm.The 84.6% of the patients with significantly higher concentration of aspartyl proteinase from the cervical flora had associated infection between mixed bacterial flora, *Gardnerella vaginalis* and *Trichomonas vaginalis*. Likewise, 85.7% of patients with significantly higher concentration of aspartyl proteinase in vaginal samples had associated infection with mixed bacterial flora, *Gardnerella vaginalis* and *Trichomonas vaginalis* which contributes to the assumption of possible synergistic activity among these infectious agents.

Through the conduction of this study, we desrcibed a metabolic activity of the infectious organisms and its effects on gynecological cytology. We showed that metabolic activity and metabolic products of the infectious agents may have a significant roles in clinical presentations and pathogenesis of the cervical diseases. Significance of the microorganisms can not be opposed and understanding of their virulence factors has an important role in prevention, treatment and outcomes of the gynecological infectious diseases.

5. ACKNOWLEDGEMENTS

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