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Autofluorescence of body fluids in early diagnostics of cervical inflammatory diseases

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Abstract *Ureaplasma urealyticum* is one of the most common sexually transmitted infections. Untreated infection caused by this bacteria in women leads to inflammatory diseases of the cervix. Women with a higher age have probably a higher risk of incidence of inflammatory problems and cervical dysplasia. Fluorescence profiling analysis is a fast and sensitive method that is also suitable for the analysis of complex mixtures. Urine monitoring based on synchronous fluorescence spectra processed into concentration matrices shows differences in the urine composition of healthy individuals and patients with different diseases. It has been found increased fluorescence intensity in the 400-420 nm excitation wavelength region in patients with different diagnoses of cervix. This findings has been studied in correlation with the positivity for the presence of *Ureaplasma urealyticum* in the sample of cervix swab.

Key words *Ureaplasma urealyticum*, Cervicitis, Cervical dysplasia, Fluorescence spectrophotometry, Synchronous spectra, Concentration matrices

1. INTRODUCTION

Mycoplasmas are the smallest wild organisms capable of reproducing themselves. Sixteen of the more than 200 species known to mycoplasias are found in the human body, but only six of them are pathologically important: *Mycoplasma pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Mycoplasma ferments*, *Ureaplasma parvum*, and *Ureaplasma urealyticum*. Because of their increased affinity for mucosal epithelial cells, they occur mainly in the mouth, upper airways and distal parts of the urogenital tract. The most widespread potentially pathogenic mycoplasma isolated from the urogenital tract of men and women is ureaplasma. It is a spherical or cocobacillary bacteria with a diameter 0.2 - 0.3 μm, occurring mostly extracellulary, although they can also penetrate inside the cells and spread through the bloodstream to organs and tissues. Among prokaryotes, they excel in the absence of a cell wall (Lanao and Pearson-Shaver, 2019).

2. UREAPLASMA UREALYTICUM

Ureaplasma urealyticum is one of the most common sexually transmitted infections, associated with urogynecological diseases and infertility, non-gonococcal urethritis, orchitis, epididymitis, prostatitis and bronchopulmonary dysplasia in neonates. However it can be also isolated in asymptomatic patients. Ureaplasma by its action in the female urogenital tract can cause inflammatory diseases of the cervix and cervical dysplasia. Elderly women and immunocompromised patients with untreated ureaplasma are at a higher risk. In most cases, their role in any particular pathology cannot be predicted, due to the high degree of colonization among healthy individuals. An infection by this bacteria also affect the formation of kidney stones and may increase the risk of premature birth. They may occur as commensals or as pathogens, depending on their parasitic uptake of essential metabolites from their hosts, such as fatty acids, amino acids, cholesterol, and nucleic acid precursors (Combaz-Söhnchen and Kuhn, 2017). The treatment of ureaplasma infection is complicated because of the high level of resistance to many commonly prescribed antimicrobials. Due to the absence of cell wall, they are resistant to all beta-lactam and glycopeptide antibiotics and the lack of de novo synthesis of folic acid causes resistance to sulfonamides and diaminopyrimidines. Antibiotics belonging to the classes of fluoroquinolones, tetracyclines, chloramphenicol, and macrolides are best suited for the treatment of this infection (Beeton and Spiller 2016). *Ureaplasma* is known in laboratory diagnostics as a strain that is difficult to cultivate due to its size, absence of cell wall and very slow growth. Since it is not possible to distinguish between *Ureaplasma* and *Mycoplasma*

hominis in the culture itself, further methods are needed. In practice, Ureaplasma DNA amplification by PCR (Polymerase Chain Reaction) is used to detect *Ureaplasma urealyticum*, which is more sensitive to ureaplasma diagnosis than culture. Currently, PCR is the most widespread method for its detection. Real-time PCR (qRT-PCR), which detects and quantifies products simultaneously with amplification, is preferred to classical PCR. The improved specificity of qRT-PCR is due to the use of a third oligonucleotide probe that binds to the target sequence. The use of a labeled probe minimizes the detection of unwanted amplicons. Since it is not necessary to maintain a viable organism for nucleic acid detection, sampling, handling, and transport are easier than in the case of cultivation because no special transport medium is needed (Waites et al., 2012).

3. FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is a fast and sensitive method that enables non-contact and non-destructive data to be obtained quantitatively (according to the intensity of radiation) and qualitatively (according to wavelengths) of the analyzed system and its components. The cells contain molecules whose structure, after excitation by UV or Vis radiation of a suitable wavelength, emits radiation - fluorescence. Substances that possess the property of autofluorescence are endogenous fluorophores. The most important endogenous fluorophores are aromatic amino acids, flavin or pyridine coenzymes. The changes that occur in cells and tissues during physiological and/or pathological processes are also reflected in the amount and distribution of endogenous fluorophores. Therefore, analytical techniques based on autofluorescence monitoring can provide information about the morphological and physiological state of cells and tissues. Fluorescent monitoring defines the metabolic profile of the system. Profiling and fingerprinting belong to strategies without the need to identify specific compounds. The system is monitored as a whole and the profiles are compared to a defined standard (blood, urine of a healthy individual). Since fluorescence methods can detect changes at the molecular level even at low concentrations, they are suitable screening and diagnostic methods. For autofluorescence analysis, specimens do not need to be specially prepared or modified. Autofluorescence differs from fluorescent signals caused by exogenous fluorophores, which are found in the body either as a product of a pathological process, a drug or intentionally applied fluorescent probes (Monici, 2005). Different types of spectra can be used for fluorescence sensing, most commonly emission, excitation and synchronous, with constant wavelength difference, expressed as the dependence of fluorescence intensity on the excitation wavelength. The three-dimensional fluorescence analysis allows an analysis of the multifluorescent system. Urine is an inviting diagnostic material due to non-invasive collection and reflects the health of the organism. A disadvantage is the concentration variability of the individuals and the influence of exogenous factors such as diet or drugs. The problem of concentration urinary diversity was solved by the introduction of fluorescent concentration matrices (Kušnír et al., 2005). Urine is a multifluorescent system in which the individual fluorophores occur in orders of varying concentrations. Non-linear dependence of fluorescence intensity on fluorophore concentration and quenching of fluorescence intensity due to high fluorophore concentration will cause undiluted urine not to collect the highest concentration fluorophores in a given sample, therefore the urine sample must be diluted by geometric order. The spectra, when aligned in space according to increasing dilution, create a contour map (concentration matrix). The concentration matrix is a perpendicular projection to the plane bounded by the excitation wavelength and the negative decimal logarithm of the volume fraction of the urine sample pφ (as pH). The 3-D graph thus generated represents the relationship between fluorescence intensity, excitation wavelength, and urine dilution. Concentration matrices provide information on total urine concentration, urinary fluorescence intensity, and fluorescence of individual fluorophores. The excitation wavelength (x coordinate) characterizes the qualitative composition of the fluorophores, pφ reports the urine density (y coordinate) and the fluorescence intensity (z coordinate) is a quantitative characteristic of the fluorophores. The contour shape shows the interactions between fluorophores. The value of one contour line characterizes 5% of the total fluorescence. The mean pφ of urine in healthy subjects is 2.1-2.4. Values above 2.4 indicate concentrated urine (Figure 1A) and values below 2.1 correspond to diluted urine (Figure 1B) (Birková et al., 2007; Dubayova et al., 2015; Píš et al., 2011). Fluorescent concentration matrices illustrate the quantitative and qualitative composition of urine.

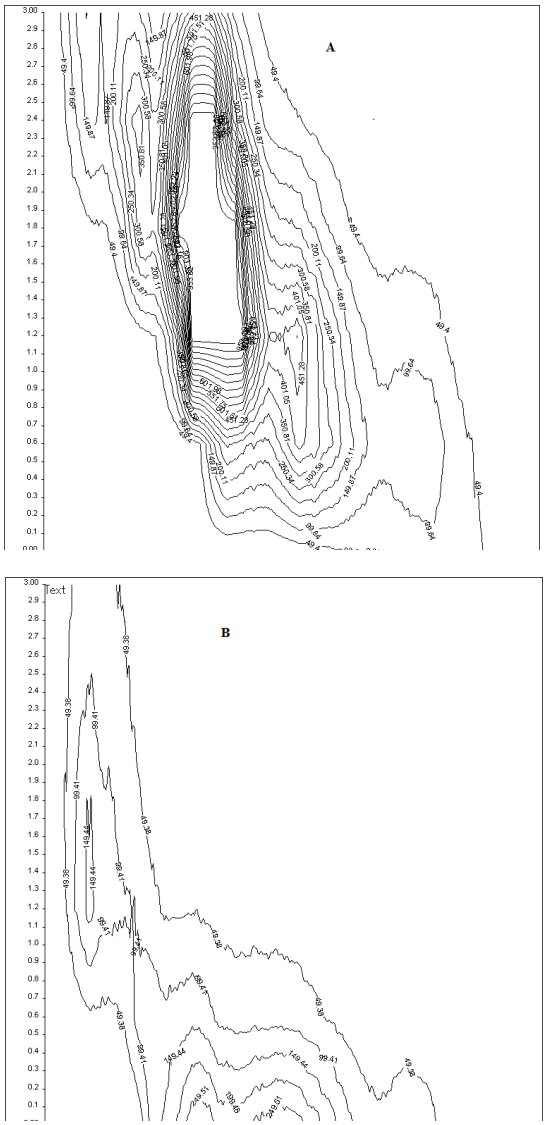

Figure 1: Comparison of urine concentration matrices of different concentration. A concentrated urine (pφ = 3); B very low density urine (pφ = 1,5)

4. AIMS

The aim of this work is to apply fluorescence profiling analysis to identify changes in its composition of patients with various cervical

pathologies that are associated with *Ureaplasma urealyticum* infection.

5. MATERIAL AND METHODS

5.1 Set of patients

The experimental group consisted of patients diagnosed with inflammatory diseases of the cervix (cervicitis), and in some of them already with cervical dysplasia, diagnoses N72, N87, N87.2, N87.9 and a patient with cervical cancer. The group consisted of 58 women aged 16 - 55 years, the average age was 33 years. Of the experimental group, 44 patients had dg. N72, 15 were positive for *Ureaplasma urealyticum*, 18 negative and 11 positive for other pathogens. Set of patients with dg. N87 consisted of 7 women, 3 positives for Ureaplasma urealyticum, 1 negative and 3 positive for other pathogens. In the file with dg. N87.9 were 5 patients, 2 positives for *Ureaplasma urealyticum* and 3 positives for other pathogens. With severe cervical dysplasia, we had one patient negative for *Ureaplasma urealyticum*. In our study, we used healthy women as controls to find no sexually transmitted diseases or their agents. The patients expressed their consent to the anonymous use of the results for scientific purposes by their signature.

5.2 Biological material

Cervical swab and morning urine of patients who have been diagnosed with inflammatory diseases of the cervix.

5.3 Detection of *Ureaplasma urealyticum*

The cervical smear DNA was isolated with a QIAamp DNA Mini Kit (Qiagen) with an automatic QIAcube isolator (Qiagen). DNA was analyzed by real - time PCR for the presence of *Ureaplasma urealyticum* and *Ureaplasma parvum* kit AmpliSens® Ureaplasma spp.-FRT on a Rotor Gene 3000 (Qiagen). Positive samples were verified by PCR for the presence of *Ureaplasma urealyticum*.

5.4 Urine analysis

Strip analysis of urine: semi-quantitative detection of pathological constituents of urine by Dekaphan strips.

Fluorescent urine analysis: urine samples were centrifuged at 10,000 rpm for 10 min. Samples (1 ml) were diluted 1: 3 with distilled water in a geometric series, from undiluted to 1000 dilution. The samples were measured in a quartz cuvette (1 cm) with a volume of 3.5 ml. For fluorescence analysis, we used a Perkin Elmer Model LS 55 fluorescence spectrophotometer. We measured the following parameters: excitation/emission slit 5/5 nm, scanning speed of monochromators 20 nm/s. We recorded a simple synchronous spectrum with a constant wavelength difference $\Delta \lambda = 30$ nm expressed as a dependence of fluorescence intensity on excitation wavelength in the range of 250 - 550 nm in the FLWinlab program. Concentration matrices were generated from the measured spectra.

6. RESULTS

The urine concentration matrix reflects its composition not only in terms of the amount of water (urine concentration) but also in the presence of fluorescent metabolites. To compare the spectra of the individual urines, it is necessary to select the dilution in which the analyzed fluorophore has a maximum or is located in a linear zone. A patient with cervical cancer had a significantly altered concentration matrix in the 400-420 nm range. To compare the fluorescence profiles (Figure 2), we selected the spectra at the dilutions shown in figure 3 marked with a blue line. The fluorescence above 1000 a. u. is out of detector measuring range. Pterins, kynurenine, folic acid fluoresce in this zone.

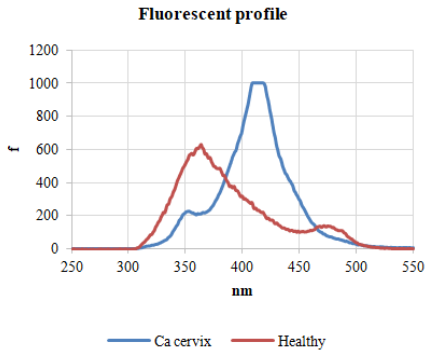

Figure 2: Comparison of fluorescence profile of a healthy woman and a patient with cervical cancer

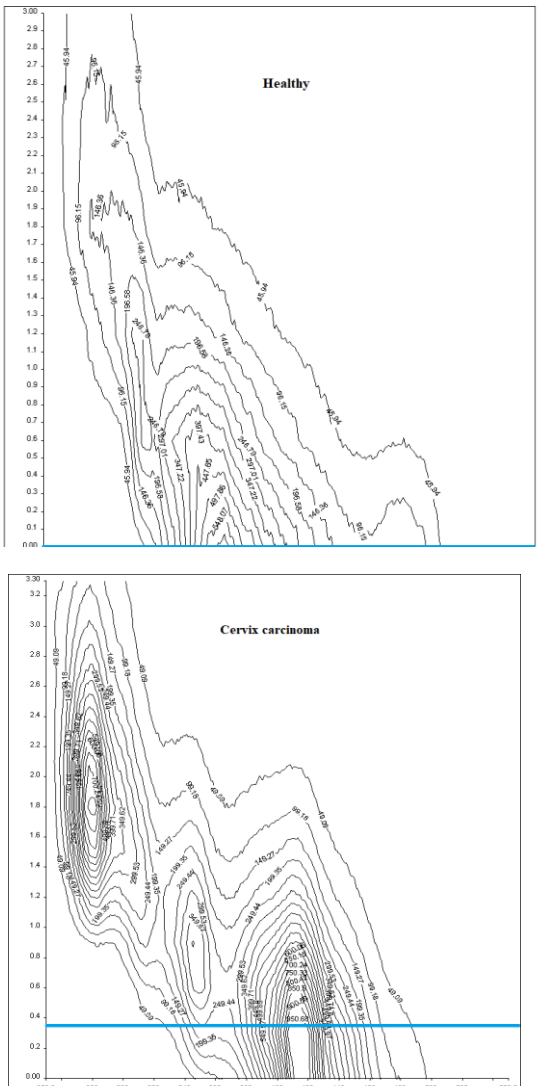

Figure 3: Comparison of concentration matrices. The blue line show the dilution at which fluorescence profile were compared

The fluorescence profile is more illustrative for comparison than the matrix, so in the following, we only compare the fluorescence profiles of urine dilutions that correspond to the fluorescence maximum of a given region. We focused on this area and compared the fluorescence profiles of patients diagnosed with N72 (Inflammatory Cervical Disease), N87.0 (Mild Cervical Dysplasia), N87.2 (Severe Cervical Dysplasia), N87.9 (Unspecified Cervical Dysplasia) related to the (in) presence of *Ureaplasma urealyticum*. This bacterium has urease activity, i.e. it produces ammonia and increases the pH of the environment. For this reason, we also focused on the pH value of urine. A patient diagnosed with N87.2 also has increased fluorescence intensity in the area (Figure 4).

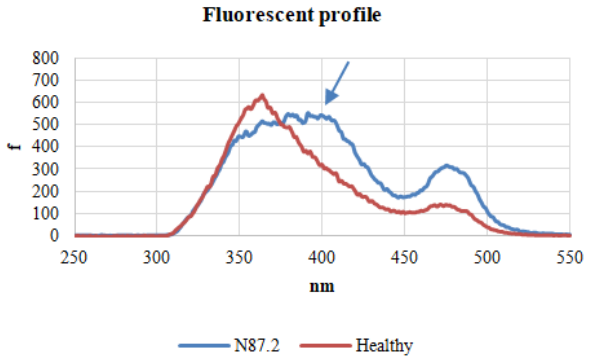

Figure 4: Comparison of the fluorescence profile of a healthy woman and a patient with severe cervical dysplasia

From the group of patients with dg. N87.9 we selected a patient that was positive for Candida albicans. The patient used folic acid, which fluoresces in the monitored zone of 400-420 nm. We wanted to avoid increased intensity due to exogenous fluorophore. As shown in Figure 3, the fluorescence profile does not differ from that of a healthy woman. The urine pH was 6. In Figure 5 there is also a profile of a patient who, in addition to positive for *Candida albicans*, also positive for *Ureaplasma urealyticum*, a urine pH of 7. The fluorescence profile has a peak at 405 nm. A patient with a positive effect only on *Ureaplasma urealyticum* has a similar profile, urine pH is also 7.

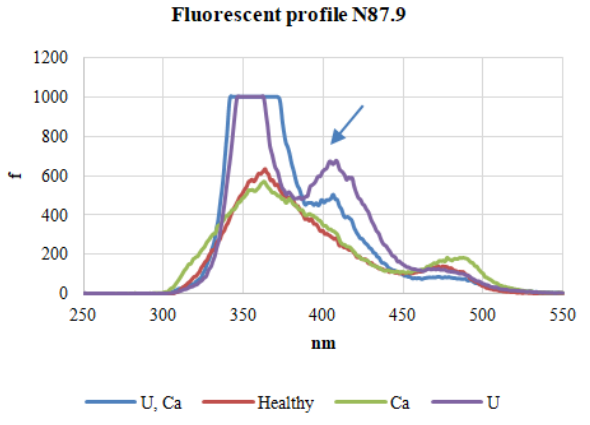

Figure 5: Comparison of fluorescence profile of patients with dg. N87.9

In the diagnosis of N72, we compared the fluorescence profiles of patients that were positive for Ureaplasma urealyticum, but we focused on the different ages of the patients. The fluorescence profile at 400 nm is very similar, except for a slight change (Figure 6). Although the same pathogen is present in both patients, the effect of *Ureaplasma urealyticum* in the elderly patient was already reflected in the pH change (pH = 7), whereas in the younger patient the increase in pH did not occur (pH = 6). The risk of developing inflammatory diseases and precancerous conditions is significantly higher in elderly patients since the negative effect of *Ureaplasma urealyticum* is believed to persist in the body longer than in younger women.

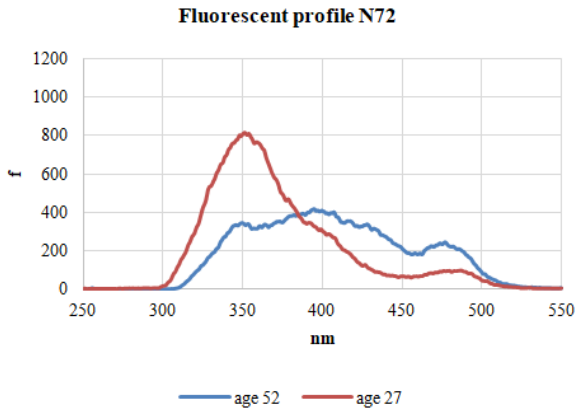

Figure 6: Comparison of fluorescence profile of patients with dg. N72

7. CONCLUSION

Fluorescent techniques are suitable for producing metabolic profiles because of their speed, efficiency, and sensitivity. Metabolic profiling is one of the modern techniques for the analysis of biological fluids. By comparing the fluorescence profiles of the healthy and the ill, it is possible to define certain differences that could be used for screening or diagnosis. The coordinates of the fluorescence spectra, the intensity of the fluorescence and the course of the contour lines create a unique fingerprint image. Fluorescence analysis revealed interesting fluorescence changes in inflammatory diseases of the cervix and precancerous conditions at a wavelength of 400 nm. We will continue with identification of other compounds that emit fluoresce in this zone. The size of the experimental set was not sufficient for statistical processing as the aim of the work was to point out the monitoring of urine by fluorescence analysis.

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