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Characterisation of protein composition and detection of IgA in cervicovaginal fluid by microchip technology

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ABSTRACT

In this paper the application of microchip electrophoresis to examine the protein profile of cervicovaginal fluid and the detection of IgA heavy and light chains is presented. This method is a fast growing field of technology and ensures high-speed analysis requiring only microliters of sample. Proteins with wide range of molecular masses could be separated within 1 min. Cervicovaginal specimens of healthy women showed a complex protein pattern-containing several peaks in the 15–70 kDa region. sIgA is considered to be an important protein constituent of all mucosal surfaces. Detection of sIgA in cervicovaginal samples was achievable by microchip technology. Under reduced circumstances (induced by mercaptoethanol, a component of the denaturating solution) the disulfide bonds connecting IgA heavy and light chains are broken up and chains can be detected as separate peaks during electrophoresis. In 82.5% of the cases only the light chain of IgA could be detected in the clinical samples. The intact IgA heavy chain could be demonstrated in only 12.5% of the cases. Based on our data some conclusions were provided about the correlation of these patterns with the age of patients, pH of the cervicovaginal fluid, operations performed before sample collection and usage of oral contraceptives.

1. Introduction

Cervicovaginal secretions have complex composition-containing glycoproteins, free carbohydrates, proteins and cytokines, although this list is not exhaustive. Vaginal fluid contains free mono- and oligosaccharides (mainly glucose, mannose and glucosamine) in relatively high concentration compared to glycoproteins. Protein components of the vaginal fluid are partly identical to those of known plasma proteins (albumin, transferrin, immunoglobulins, etc.), but it also contains unique proteins. The protein composition of the vaginal fluid refers to the fact that it is (in part) a transudate of the plasma. The vaginal cells and the vaginal fluid do not contain identical proteins, so the vaginal cells do not contribute any portion of the vaginal fluid [1].

The predominant antibody isotype on mucosal surfaces and in secretions is usually IgA, in the form of secretory IgA (sIgA). It is produced by the local plasma cells and excreted in the secretions. The main role of sIgA is the protection of mucosal surface and insurance immunity against pathogens [2].

Although the genital tract is considered to be a component of the mucosal immune system, it possesses special properties. In the cervicovaginal secretions the dominance of secretory IgA could not be proved. In vaginal fluid IgG rather than IgA is the dominant isotype [3]. Vaginal secretions contain locally produced and plasma-derived immunoglobulins. IgG-secreting cells are presented in the endocervix [4]. The high percentage of the IgG1 isotype in the cervicovaginal fluid is related to the diffusion and/or receptor-mediated transport of IgG from the plasma into the lumen. Besides the dominating IgG fraction sIgA is also present in the vaginal fluid. A part of vaginal IgA can also derive from the serum. On the other hand immunohistochemical examinations revealed the presence of IgA secreting plasma cells in the sub-epithelial layers of uterine endo- and ectocervix, fallopian tubes and the vagina [5].

Concentration of the sIgA in the vaginal fluid may be influenced by several physiological or pathological factors. Vaginal concentration of IgA was found to be significantly higher in post-menopausal women compared to fertile women and those treated with oral oestriol [6]. In fertile women the immunoglobulin levels changed during the menstrual cycle [7]. Pregnancy also has a modifying effect on the cervicovaginal IgA concentration. An increase in the

Abbreviations: sIgA, secretory immunoglobulin A; HIV, human immunodeficiency virus.

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level of class A immunoglobulins could be detected during pregnancy. IgA/IgG ratio and statistical calculations confirmed the local production of these IgA antibodies [8]. In contrast with these, the usage of oral contraception did not induce significant changes in the concentration of vaginal IgA and IgG classes [9].

Local and systemic infections (e.g. HIV [4], HSV, and *Candida* [10–13]) or changes in the composition of the vaginal normal bacterial flora (bacterial vaginosis) [14,15] may also cause changes in the concentration of IgA antibodies in the cervicovaginal fluid.

Based on the varied but often not definitive results the aim of this study was to examine the protein composition of the cervicovaginal fluid and detect the IgA heavy and light chains in the vaginal secretions of uninfected women. During this study we introduced a new, fast, non-invasive filter paper method for sample collection that was agreeable to the patients, and was able to provide sufficient sample for protein analysis. The protein content and the presence or absence of IgA heavy and light chains were analysed by microfluid chip technology. Chip electrophoresis allowed rapid separation, sizing and detection of proteins within 1 min. The resulting digital data gives the possibility for high-throughput biological studies and allows statistically valid studies to be performed.

2. Experimental

2.1. Sample collection and preparation

Participants in this study were recruited from symptomless, healthy women who visited the Gynaecological Care Unit at the University of Pécs, Hungary. The study was approved by the Ethical Committee of the Medical Faculty of the University of Pécs. All patients and controls gave informed consent. Patients were pre-, and post-menopausal women (aged from 21 to 60 years), who were interviewed and examined by a clinician. Standardised cervicovaginal sampling was done for pH determination. Forty control women were selected who did not have vaginal symptoms in the last 2 months, had pH $\leq$ 5.5, were not pregnant, were without menstrual bleeding and did not use systemic or vaginal antibiotic treatment. Three of the post-menopausal patients were receiving hormone replacement therapy. Fifteen of the fertile women used oral contraception.

The specimens were collected by the application of 0.25 cm^2 area sterile filter paper strips (Schirmer paper, Ciba Vision, Novartis International AG, Basel, Switzerland) usually used in ophthalmology for tear collection. The quantity of the cervicovaginal fluid soaked in the filter paper during 60 s was sufficient for the protein analysis by microchip technology. Paper strips were placed directly into 0.5 ml sterile Eppendorf tubes without preservative and stored at −20 °C until examination.

Before microchip examination could be performed 30 μl of sample buffer was added to each filter paper strip. This was then vigorously vortexed for 30 s and the strip removed using sterile forceps. Sample buffer contained 0.125 M Tris–HCl, pH 6.8, 10% SDS and 4% β-mercaptoethanol. All materials were purchased from Sigma (St. Louis, MO, USA).

Samples were prepared for microchip electrophoresis according to the manufacturer's instructions (see next section for details).

2.2. Microchip electrophoresis

Chip-based separation of proteins was performed using the commercially available Agilent 2100 Bioanalyzer System (Agilent Technologies, PaloAlto, CA, USA). Protein 230 Plus LabChip Kit (catalogue number: 5067-1517) were applied in the study. Microcapillaries of the protein chips were filled up with the gel–dye mix of the Kit. The gel contained a linear polymer as the sieving agent and ensured the separation of protein–SDS complexes on the basis of their molecular mass, within the 14 and 230 kDa range. Staining dye was added to the gel matrix and SDS–protein complexes were labelled on chip. LIF detection was used in the measurements.

Four microliters of prepared clinical sample was mixed with 2 μl of denaturating solution which contained β-mercaptoethanol and lower and upper internal markers for accurate sizing. Samples were boiled for 5 min, centrifuged and diluted with 14 μl of distilled water. Six microliters of each sample was loaded onto the sample well of the chip. Electrophoresis was performed three times on all samples. The ladder of the LabChip Kit containing several molecular weight markers was applied for the molecular weight determination of the unknown proteins. Evaluation of the molecular weight and quantity of the cervicovaginal proteins was performed by the Protein 230 assay software.

2.3. Detection of IgA in the clinical samples

Human IgA was purchased from Sigma Ltd. (St. Louis, MO, USA). Under reduced circumstances the IgA heavy and light chains are separated and can be detected as separate peaks by electrophoresis. According to available data in the literature the molecular mass of the IgA light chain is about 24–28 kDa, while the heavy chain is ca. 55–70 kDa.

0.5 mg/ml stock solution of IgA was prepared. This solution and further dilutions (2×, 5× and 10×) were sent for microchip electrophoresis.

Presence of IgA heavy and light chains in the cervicovaginal fluid samples was detected by addition of IgA solutions to the clinical samples. One microliter of the IgA solution was added to 3 μl of the samples and measured in parallel with the untreated sample. The peaks corresponding to IgA light and heavy chains were proportionally higher after the addition of different concentrations of IgA solutions.

3. Results

3.1. Characterisation of the protein content of the cervicovaginal fluid

The quantity of the cervicovaginal proteins obtained from the filter paper strips was sufficient for the analysis of the protein composition by microchip technology. A typical protein profile of cervicovaginal fluid obtained by microfluid chip technology is illustrated in Fig. 1. As it can be seen in this figure, this method ensured quantitative data within 45 s. The protein content of the cervicovaginal fluid showed a complex pattern. Several protein peaks could be detected in the molecular weight region from 15 to 70 kDa, respectively. Most of the proteins were present in this molecular mass range. In the higher molecular weight region only a few proteins could be detected and in low quantity. Baseline separation of the protein peaks could not be achieved in our experiments, which can be explained by the complex composition of these clinical samples and presence of numerous proteins within them.

In 15 cases (37%) the concentration of the proteins was similar in each molecular weight region (from 15 to 70 kDa) and no dominating protein could be detected. In 23 cases (57%) the electrophoretic patterns were dominated by the protein peaks of the 55–66 kDa region. This range contained at least two, but usually 4–5 protein peaks. These proteins were presented in much higher concentration in the clinical sample (70–80%), than other proteins (Fig. 1). In 2 cases (5%) protein peaks could only be detected in this 55–66 kDa region.

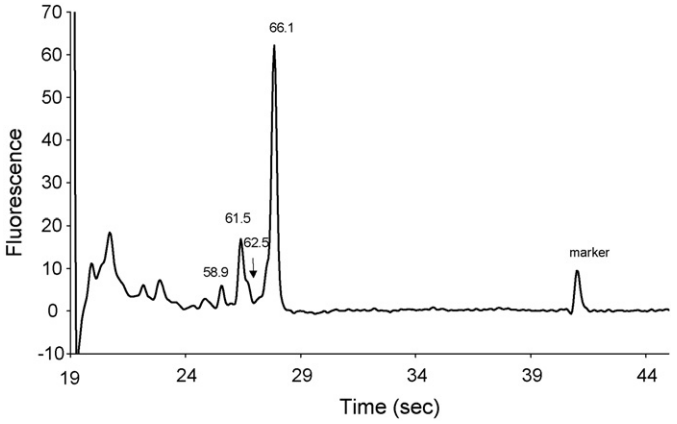

Fig. 1. Microchip-based electropherogram of cervicovaginal fluid obtained from a healthy woman. The complex protein pattern contained several major and minor proteins in the molecular weight region from 15 to 70 kDa. Dominance of the 55–66 kDa region can be seen in the figure. Molecular mass of the characteristic peaks of this region is indicated in the figure. Peak of the high molecular mass internal standard (upper marker) can be seen at ca. 43 s. Experiments were performed in Protein 230 LabChips of the Agilent 2100 Bioanalyzer system according to the protocol provided by the manufacturer.

3.2. Detection of IgA light and heavy chains in the clinical samples

The stock solution of IgA and its 2, 5 and 10 times dilutions were prepared and sent for microchip electrophoresis. The pattern of the concentrated sample is demonstrated in Fig. 2. Light and heavy chains of IgA could be detected with molecular weights of 26.1 and 70.9 kDa, respectively. Beside these peaks an additional peak was also presented in the pattern with molecular weight of 32.4 kDa, respectively. This intermediate peak appeared in all measurements and seemed to be sensitive to the addition of IgA to the control sample. The occurrence of this additional peak may be explained by the degradation of some protein chains under reduced circumstances.

Presence of IgA light and heavy chains in the clinical samples was analysed by the addition of different concentrations of IgA. The proportional increase in the height and area under the curve of certain peaks after addition of IgA proved the presence of IgA chains in the specimen. The interpretation of patterns for IgA heavy chains was much easier to perform as only a few smaller peaks were detected in this molecular weight region. Fig. 3 depicts a typical pattern achieved when both heavy and light chains are detected in a clinical sample and these peaks are marked with arrowheads on the diagram. In Fig. 4 a cervicovaginal protein pattern is demonstrated, in which only the light chain of IgA is detectable.

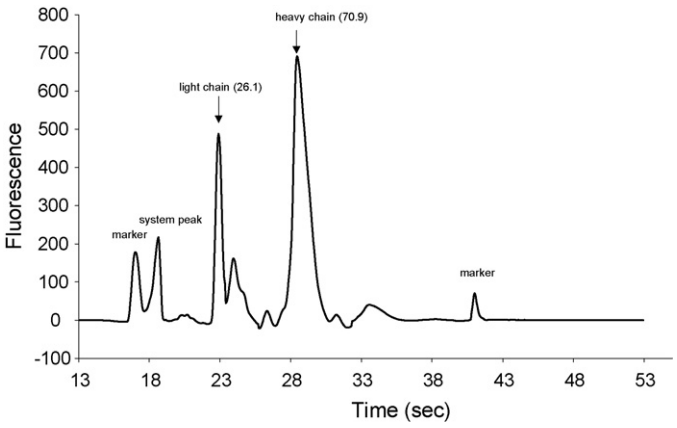

Fig. 2. Electropherogram of human IgA (0.5 mg/ml) obtained by microfluid chip technology. Light and heavy chains of IgA with apparent molecular masses of 26.1 and 70.9 kDa are signed by arrowheads in the figure. An additional peak with molecular mass of 32.4 kDa was also presented in the pattern.

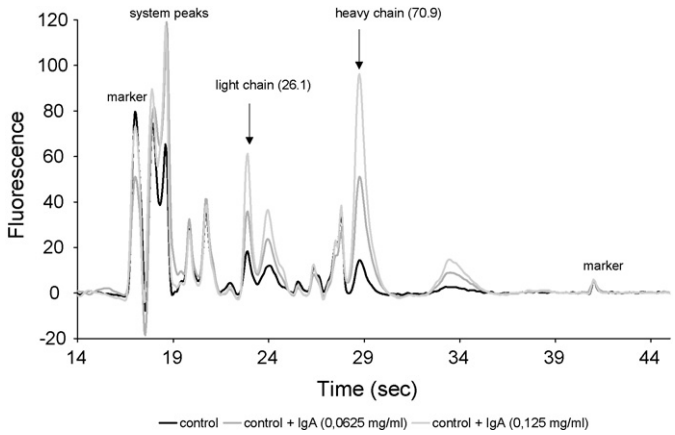

Fig. 3. Microchip-based electropherogram of a cervicovaginal sample containing IgA heavy and light chains. Pattern of the cervicovaginal fluid under control circumstances is presented by black line in the figure. Presence of IgA heavy and light chains in the sample was checked by addition of different concentrations (0.0625 mg/ml and 0.125 mg/ml) of IgA solution to the sample. Patterns of these mixtures are presented by grey lines in the figure. Proportional increase in the heights of the peaks corresponding to heavy and light chains after the addition of IgA is presented in the figure. Peaks of the heavy and light IgA chains were signed by arrowheads. Peak of the low and high molecular mass markers can be seen at ca. 17 and 43 s. Systems peak arisen from the endosmotic flow are presented at ca. 18 and 19 s. Experiments were performed in Protein 230 LabChips of the Agilent 2100 Bioanalyzer system according to the protocol provided by the manufacturer.

Based on this method the clinical samples could be divided into three distinct groups. In group 1, both the heavy and light chains of IgA could be demonstrated by microchip electrophoresis (12.5%). The second group contained the biggest percentage of samples (82.5%) where only the light chain was detectable. In the remaining group neither the light, nor the heavy chain could be observed (5%).

Characterisation of these groups in respect to clinical aspects is summarised in Table 1. Age of the patient (including mean age and standard deviation), pH of the cervicovaginal fluid, any operations performed before sample collection and usage of oral contraceptives were the aspects detailed. As it can be observed in the table,

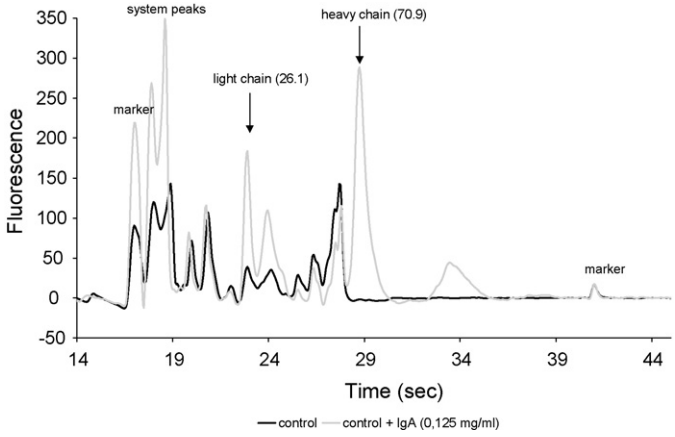

Fig. 4. Protein pattern of a cervicovaginal sample containing only the light chain of IgA. Electropherogram of the control sample is presented by black line, while the pattern obtained after the addition of IgA (0.125 mg/ml) is demonstrated by grey line. Peaks corresponding to the heavy and light IgA chains were signed by arrowheads. Increase in the height of the light chain peak, and absence of the heavy chain is presented in this figure. Peak of the low and high molecular mass markers and the system peak are demonstrated on the figure.

Table 1
Based on the protein composition of the cervicovaginal samples the 40 women examined in this study were classified in three groups

	Age	pH	Operation	Oral contraceptives
Group 1 (5/40)	47.2 ± 7.4	4.3 ± 0.2	20%	0
Group 2 (33/40)	33.5 ± 8.2	4.3 ± 0.3	27%	39%
Group 3 (2/40)	29.0 ± 9.9	4.3 ± 0.14	0	100%

We indicated in brackets the number of cases within each group per total cases. Mean age (and standard deviation) of the patients, pH of the cervicovaginal fluid, percent of operated patients, and percent of patients receiving oral contraception in different groups of healthy women. In group 1, both the heavy and light chains of IgA could be detected, in group 2 only the light chain, and in group 3 neither chain was detectable by microfluid chip technology. Most of the healthy patients belonged into the second group.

the mean age of group 1 patients was higher than in the other two groups, with the lowest mean age being seen in group 3. This group contained only two patients and it would therefore be beneficial to conduct further examinations utilising a younger patient group. As a preliminary result it can be concluded that this protein pattern was never observed in post-menopausal patients. There were overlaps between groups 1 and 2 (e.g.: group 2 contained also a 58 years old patient) that might be explained by individual biological differences and by the fact that each patient can be characterised by a different menopausal status at the same age. As a consequence the difference between the mean ages of these groups was remarkable.

The pH of the cervicovaginal fluid was identical in the three groups.

Post-operative status did not influence the results, and the ratio of the operated patients was similar in the first two groups. Effect of oral contraceptives on the IgA content may be more interesting as none of the patients in the first group were receiving oral contraceptives, whilst almost 40% in the second group were.

4. Discussion

Several methods have been used for sample collection and examination of the protein content of cervicovaginal fluid samples. The application of filter paper strips to gain vaginal fluid is a non-invasive, fast method that is easy to perform during the normal gynaecological examination. Schirmer paper strips, applied in ophthalmology for tear collection were used in our experiments. Absorption of proteins onto the paper strip and their elution has previously been examined. It has been proven that this procedure may have some influence on the concentration of certain proteins [16,17], but in all documented cases proteins remained detectable. Hoshino et al proved that sIgA could be detected in the fluid soaked onto the Schirmer paper [18].

The protein composition of cervicovaginal fluids has already been analysed by different methods including gel electrophoresis, Western blot [14], immunodiffusion [6] and ELISA [13]. This application of microfluid chips is a new opportunity that has many advantages. The technique ensures high speed and comprehensive analysis of complex biological samples. The main benefit of this technique is the rapid separation which produces a protein profile within a few seconds. These integrated analytical systems utilise small sample volumes, produce faster responses and allows parallel analyses with minimal cross-contamination and automation capabilities [19].

The lab-on-a-chip architecture employs capillary gel electrophoresis to separate proteins on the basis of their molecular weights. Electrophoretic analysis of cervicovaginal fluid demonstrated a complex protein composition. Microfluid chip technology was suitable for detection of IgA within these samples. IgA could be present as monomer, dimer or trimer form in the cervicovaginal secretion before treatment with reducing reactants. Here it has to be emphasized that classes, subclasses allotypes and isoallotypes of IgA cannot be identified using this technique.

Cauci et al. [14] described that in 70% of healthy women IgA heavy chain can be detected in the cervicovaginal secretions. In our microchip analysis the heavy chain of IgA could only be detected in 12.5% of the patients. In most of our patients only the light chain was detected. Lack of the heavy chain within these secretions may be explained by the degradation of this protein by proteolytic enzymes [14].

Based on our electrophoretic analysis the IgA heavy chain was detected in the cervicovaginal secretions of older patients. Within this age group secretory IgA might play an important role in the local defence against infection. It is known from literature data [20] and from our examinations [21] that in post-menopausal women the *Lactobacilli* are either absent from the normal vaginal flora or present in much lower quantities than in younger women. This situation cannot be influenced by hormone replacement treatment. Normally *Lactobacilli* play a significant role in the protection of the genital tract against infections. Despite this fact infections do not occur more often within this age group [22]. Perhaps the presence of intact IgA in an increased percentage may explain (at least partly) the low incidence of infections.

In our experiments we could not find any correlation between pH of the vaginal fluid (within the normal range), surgical procedure performed on the patient and presence of IgA heavy chain. Oral contraceptive use seemed to correlate with the presence of IgA in the cervicovaginal samples. This finding requires further investigation. Effect of patients' age on the presence of IgA heavy chain in the cervicovaginal fluid is demonstrated in this study. Fast detection and low sample volume required by microchip makes this technology applicable for everyday practice.

Based on our data we conclude that microchip electrophoresis is suitable for the characterisation of cervicovaginal protein profiles and for the detection of IgA in these samples. With results available within minutes after examination this technique provides the potential to promote care and rapid treatment of patients.

Correlation of IgA heavy chain with physiological and pathological processes requires further investigation.

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