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A candidate serum biomarker for bladder pain syndrome/interstitial cystitis†,‡

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Abstract

Reliable diagnostic markers for Bladder Pain Syndrome/Interstitial Cystitis (IC) currently are not available. This study evaluated the feasibility of diagnosing IC in humans and domestic cats from the spectra of dried serum films (DSFs) using infrared microspectroscopy. Spectra were obtained from films from 29 humans and 34 domestic cats to create classification models using Soft Independent Modeling by Class Analogy (SIMCA). Ultrafiltration of serum improved discrimination capability. The classification models for both species successfully classified spectra based on condition (healthy/sick), and a different set of masked spectra correctly predicted the condition of 100% of the subjects. Classification required information from the 1500–1800 cm⁻¹ spectral region to discriminate between subjects with IC, other disorders, and healthy subjects. Analysis of cat samples using liquid chromatography–mass spectroscopy revealed differences in the concentration of tryptophan and its metabolites between healthy and affected cats. These results demonstrate the potential utility of infrared microspectroscopy to diagnose IC in both humans and cats.

Introduction

Painful Bladder Syndrome/Interstitial Cystitis (IC) is an idiopathic chronic pain syndrome; its causes remain unknown, and no generally effective treatments are available.¹ A syndrome comparable to IC, called feline IC (FIC) also occurs in domestic cats. No well accepted diagnostic test for (F)IC currently exists. In addition to its clinical utility, a sensitive and specific diagnostic marker for IC could greatly facilitate studies of the pathogenesis and treatment of this syndrome. Potential diagnostic markers for IC with varying sensitivity and specificity have been described in urine, serum, and bladder tissue.² Keay *et al.*³ recently summarized current research regarding biomarkers for IC in urine. Although the antiproliferative factor and decrease in heparin-binding epidermal growth

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factor have shown some potential as diagnostic markers, they currently are not available clinically, and appear to be state-dependent markers of symptom severity.⁴

Vibrational spectroscopic methods such as Fourier transform-infrared (FT-IR) spectroscopy and Raman spectroscopy are emerging as powerful techniques in biomedicine. These methods permit rapid, high-throughput, non-destructive analysis of biological samples because the IR absorption spectrum provides a characteristic ‘fingerprint’ of substances such as lipids, proteins, nucleic acids, polysaccharides, and phosphate-carrying compounds present in the sample.⁵

Combining infrared microspectroscopy (IRMS) with multivariate statistical approaches such as principal components analysis (PCA) has considerable potential for use in routine clinical diagnostic analyses. For example, Sakudo *et al.*,⁶ recently used IRMS coupled to PCA and soft independent modeling of class analogy (SIMCA) to develop multivariate models capable of discriminating patients with chronic fatigue syndrome (CFS) from healthy control subjects. They correctly predicted 54 of 54 healthy subjects and 42 of 45 (93%) of CFS patients from spectra of masked serum samples. Hsieh *et al.*⁷ reported the application of Raman microspectroscopy for IC diagnosis and found that tryptophan and phenylalanine discriminated the severity of IC symptoms.

The purposes of this study were to evaluate the feasibility of using IRMS to diagnose IC, investigate the presence of a serum biomarker for IC in humans and domestic cats with feline IC (FIC), develop multivariate classification models for the rapid diagnosis of (F)IC from serum, and to identify chemical compounds that might be responsible for distinguishing healthy from affected subjects using liquid chromatography–mass spectroscopy (LC–MS).

Experimental

Biomarker development approach

The initial phases of biomarker development described by Hewitt *et al.*⁸ were adapted for use in the current studies. All experiments were performed in compliance with the relevant laws and institutional guidelines; the Institutional Review Board and Institutional Animal Care and Use Committee of The Ohio State University approved the human and animal experiments, respectively. Written informed consent was obtained for all samples obtained from human subjects.

Domestic cats—Serum samples were provided by the Department of Veterinary Clinical Sciences of The Ohio State University (Columbus, OH) from healthy cats (5 male, 6 female), cats with FIC (11 male and 11 female), and cats with other disorders. Cats with FIC were obtained as donations from veterinary clients, and the diagnosis of FIC was confirmed as previously described.⁹ Healthy adult cats matched as closely as possible for age obtained from licensed vendors were used as controls. Adult cats with disorders unrelated to IC were used to test the specificity of the method using serum obtained from the clinical biochemistry laboratory of the Ohio State University Veterinary Teaching Hospital. Samples were obtained from healthy cats and cats with FIC under baseline vivarium conditions, and after imposition of an acute stressor previously shown to activate the stress response system of cats.¹⁰

Humans—Serum samples were obtained from the Harvard Urological Diseases Center (Boston, MA) and from the Urology Division at The Ohio State Medical Center to permit comparisons between healthy subjects, those with IC, and those with other urological disorders. All IC patients satisfied either the criteria for the diagnosis of IC described by the

National Institute for Diabetes and Digestive and Kidney Diseases (NIDDK),¹¹ or the more recently accepted clinical criteria.¹ Healthy control subjects were chosen based on the absence of historical or physical evidence of lower urinary tract dysfunction.

Sample preparation and analysis—Serum was separated by centrifugation and samples were immediately stored at -20°C to maintain their integrity until analysis. Serum (1 : 1 or 1 : 5 v/v serum : water) solutions ($\sim 5\ \mu\text{L}$), complete or fractionated by centrifugation through a cellulose membrane of 10 000 nominal molecular weight limit (NMWL) at $16\ 000\times g$ for 10 min using a microcentrifuge (Model 5415R, Eppendorf, Westbury, NY), were placed ($0.5\text{--}1\ \mu\text{L}$) onto SpectCONC-IR or SpectRIM (Tienta Sciences, IN) microscope slides (circular area of $3.8\ \text{mm}^2$) and dried at room temperature under vacuum to produce dried serum films (DSFs). Serum was fractionated by centrifugation followed by vacuum drying of $1\ \mu\text{L}$ of the filtrate over the SpectCONC-IR microscope slides.

Infrared microspectroscopy—Spectra were recorded using an infinity corrected FT-IR microscope [UMA 600 series IR microscope interfaced with an Excalibur 3100GX FTIR spectrometer (Varian, Walnut Creek, CA)], equipped with a motorized x - y stage, $4\times$ and $16\times$ objectives, and a broadband mercury-cadmium-telluride detector. The spectrometer was controlled by Resolution Pro Software (Version 3.0.5.009, Varian) that was programmed to collect spectra over the wave-number range $4000\text{--}700\ \text{cm}^{-1}$. The spectral resolution used was $8\ \text{cm}^{-1}$, and 128 spectra were co-added and averaged to improve the signal-to-noise ratio. The absorbance spectrum was obtained by ratioing the single beam spectrum against that of the gold background. Spectra were displayed in terms of absorbance as calculated from the transmittance spectra using the Resolutions Pro software.

Multivariate data analyses—Spectral differences between samples from subjects with (F)IC, subjects with other medical disorders, and asymptomatic controls were evaluated using multivariate statistical techniques to resolve spectral information of interest, and to cluster the samples according to the presence of the disease. Soft Independent Modeling Class Analogy (SIMCA) was carried out using Pirouette pattern recognition software (V 3.02 for Windows NT, Infometrix, Inc., Woodinville, WA). Sample residual and Mahalanobis distance were used for outlier diagnostics. The scores plot was used for visualization of clustering among samples (sample patterns, groupings or outliers). Coomans plots were applied to assess the classification performance of the SIMCA model by predicting class membership in terms of distance from the boundaries of the prediction model. In a Coomans plot,¹² the two axes that divide the plot into four quadrants represent the distance of each sample from a specific class (*e.g.*, IC or healthy), so that each class model is drawn as a rectangle corresponding to the critical distance ($p = 0.05$) from the class. Any sample having a distance from the corresponding class rectangle greater than the critical distance is considered to be outside the class model, and is rejected as an outlier (graphically, it is plotted outside the rectangle defining the class model). Samples plotted into the lower left quadrant of the diagram are assigned to both classes, whereas those plotted into the upper right quadrant of the diagram are assigned to a separate class.

Prediction models—The predictive accuracy of the calibration models was validated using an independent test set of samples (10 cats) representative of the classes modeled with the training set. Double-blind samples with random codes including FIC and healthy subjects were provided for diagnosis based on the spectral data and multivariate models. At least 2 of the 3 spectra of every film had to be recognized by the SIMCA models to consider the subject correctly identified. Whenever the spectra of the same film had contradictory identifications, the results would be reported as misidentified.

Liquid chromatography–mass spectrometry (LC–MS) analysis—LC–MS was used to measure levels of tryptophan and two of its metabolites, kynurenine and 5-hydroxyindoleacetic acid, in serum from 10 cats with FIC and 10 healthy cats. Analytes were separated using liquid chromatography on a reversed-phase 3.5 μm Symmetry C18 column (4.6×150 mm, Waters Corp., Milford, MA) using acidified water (0.5% trifluoroacetic acid) as the mobile phase (0.8 ml/min) for the first 8 minutes; methanol was linearly introduced from 0 to 30% until minute 20, and this concentration was maintained until the end of the analysis (minute 30). Spectral information over the wavelength range of 254–700 nm was collected on a PDA detector. Mass spectrometry was conducted on a single quadrupole ion-tunnel MS equipped with electrospray ionization (ESI) interface (Shimadzu, Columbia, MD), using selective ion monitoring under positive mode. Analytes were identified by comparison with authentic standards (Sigma-Aldrich, St Louis, MO).

Results and discussion

Infrared spectra of DSFs from cats and humans showed similar spectral profiles (Fig. 1), with no observable differences between spectra from (F)IC affected and healthy subjects. The method of preparation of the DSF had a significant impact on the reproducibility of the IR spectra. Three factors influenced the structure of DSFs: the concentration of the serum solution, the volume of sample to dry and the drying time. Sample dilution (1 : 1–1 : 5), small sample volumes (0.5–1.0 μL) per film, and short drying times (~ 4 min) provided the best results. Mechanized systems could potentially reduce the variability introduced by film preparation and drying. Collecting spectra from DSF areas with a similar appearance, roughly equidistant to the center of the film, also increased the reproducibility of the protocol.

The classification of specimens as (F)IC or healthy using dried serum films produced complex SIMCA models (>20 factors), presumably due to the presence of compounds unrelated to the disorder. For example, most serum proteins are large (65–97% represent serum albumin and immunoglobulins), whereas biomarkers often are low-molecular-weight proteins or peptides secreted into the bloodstream as a result of a disease process.^{17,18} To reduce interferences caused by large molecules that may not be related to (F)IC, films were prepared after ultrafiltration of cat serum solutions using a 10 000 molecular weight cut-off membrane. Differences in IR spectral features between films prepared with or without the ultrafiltration step (Fig. 2) were evident, with a marked decrease in some IR absorption bands in the 1400–1800 cm^{-1} region, probably due to the removal of large proteins.

The SIMCA analysis of the transformed (second derivative) IR spectra revealed specific bands that permitted the classification of samples according to presence or absence of (F)IC (Fig. 3A), with interclass distances of 4.1 and 7.9 for cats and humans, respectively. The interclass distance describes the separation between classes in multivariate space, wherein short distances indicate similarity between samples, and longer distances represent greater separation of the classes. In general, interclass distances greater than 3 suggest well separated classes.¹³ Our results thus provide evidence of chemical differences between the serum of healthy and (F)IC afflicted subjects. Spectra from cats suffering disorders other than FIC differed from cats in both the healthy control and FIC groups (Fig. 3B). SIMCA models generated using the IR spectra from membrane-filtered cat serum solutions (Fig. 3C) resulted in increased interclass distances between FIC and healthy subjects, from 4.1 to 5.9, and again correctly classified each one of the spectra.

In addition to the removal of serum constituents that may be unrelated to the syndrome, we also tested introduction of more information (classes) into the models (subject gender) to decrease the complexity of the models and increase the prediction capabilities. Inclusion of

gender into the classification models (4-class models: healthy male, healthy female, affected male and affected female) produced simpler models (fewer factors) with larger interclass distances (Table 1). These results demonstrate that IR spectra contained information about other characteristics of the subjects that could be included in the models to simplify and increase their prediction capabilities. Besides gender, other characteristics such as age, diet, or intercurrent disease that might influence serum composition could be introduced as factors to investigate their effect on the predictive power of the SIMCA models.

The SIMCA models revealed that most of the variance between (F)IC and healthy subjects could be explained by changes in IR bands in the 1500–1800 cm^{-1} range, suggesting chemical similarities of the biomarker in both species (Fig. 4-1). As shown in Fig. 4-2, the band at 1597 cm^{-1} , associated with indole ring vibrations, was one of the most important signals to discriminate healthy cats from cats with FIC, suggesting that derivatives from tryptophan may be linked to functional syndromes like (F)IC.

Based on the LC–MS analysis, serum from cats with FIC had higher concentrations of tryptophan (21% higher; %RSD: 23% (affected), 19% (healthy); $p = 0.07$) and kynurenine (23% higher; %RSD: 27% (affected), 23% (healthy); $p = 0.09$) than did serum from healthy cats, whereas those of 5-hydroxyindoleacetic acid were not different ($p = 0.45$). Interestingly, sera from patients with chronic fatigue syndrome also reportedly have comparably higher concentrations of tryptophan than do sera from healthy individuals.^{14–16} In another recent study, Hsieh *et al.*⁷ also reported that tryptophan (or phenylalanine) might serve as a biomarker to assist the diagnosis of IC in patients identified by potassium-sensitive testing using Raman microspectroscopy. Further analysis will be necessary to explore the possibility of additional compounds that also may be informative.

By using an independent set of cat serum samples, SIMCA prediction models (Table 2) correctly identified 16 out of 20 dried serum film spectra based on the condition of the subjects and gender (4-class), which permitted correct identification of the condition of the 10 subjects involved in the test from at least 1 DSF per subject, with zero misidentifications. Additionally, SIMCA models successfully classified spectra from samples obtained from women with other urological disorders. The models correctly differentiated the condition of 100% of the subjects as healthy, IC, or other urological disorder. As before, most of the discrimination of IC was associated with wave-numbers normally associated with tryptophan and pyridinecarboxylic acids.

Conclusions

This research begins to lay a foundation for a novel (F)IC diagnostic serum biomarker based on IRMS. The results provide evidence of infrared spectral differences between the serum of healthy subjects and patients with IC in two species, human and domestic cat, and suggest that chemical structures with IR absorption in the 1500–1800 cm^{-1} range may account for the discrimination. Ultrafiltration of serum solutions and specific preparation techniques of the DSF improved the performance of the protocol. The IR spectral information coupled with LC–MS could lead to the identification of even more biomarkers specific for (F)IC. The screening ability of IRMS combined with the identification capabilities of LC–MS will assist the identification of biomarker(s), and could provide information to better understand the causes of this condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Typical reflectance infrared microspectroscopy spectra of human and domestic cat serum films collected in the 4000–700 cm^{-1} region.

Fig. 2.
Infrared microspectroscopic spectra of cat serum sample before and after membrane filtration.

Fig. 3.

Coomans plots of IR microspectroscopy spectra. The vertical and horizontal lines represent the 0.05 confidence limits between groups. (A) Spectra from human serum samples, demonstrating that the healthy and affected classes of test samples did not share common multivariate space. (B) Spectra from domestic cat serum from FIC, healthy, and subjects with chronic disorders other than FIC. (C) Coomans plot of cat serum samples after membrane filtration, which increased the distance (difference) between the groups.

Fig. 4.

(1) Discriminating power plots of SIMCA models based on the infrared spectra of dried serum films (4-class models) from humans and domestic cats. The same infrared region (1500–1800 cm^{-1}) was important for classification models in both species. Letters A ($\sim 1660 \text{ cm}^{-1}$) and B ($\sim 1740 \text{ cm}^{-1}$) denote similar bands. (2) Discriminating power plot of spectra from cat serum samples after membrane filtration (2-class model).

Table 1

Interclass distances in 4-class (F(IC) condition and gender) SIMCA models using vacuum dried serum films. Number of factors in parentheses. *F*-Test confidence level: 95%

Humans	Healthy male	IC male	Healthy female
Healthy male (10)	—	—	—
IC male (11)	19.5	—	—
Healthy female (9)	11.6	6.2	—
IC female (14)	12.6	3.9	7.1

Cats	Healthy male	FIC male	Healthy female
Healthy male (8)	—	—	—
FIC male (15)	7.1	—	—
Healthy female (12)	16.8	5.7	—
FIC female (14)	17.1	5.8	4.9

Table 2

SIMCA model accuracy for identification of FIC in a masked set of domestic cat sera. Three spectra from each of two films were obtained from each serum specimen

Specimen	Class	Sex	Correctly identified films ^a		Not recognized	Wrongly identified
A	FIC	Female	2	0	0	
C	FIC	Female	1	1	0	
D	FIC	Female	2	0	0	
B	FIC	Male	2	0	0	
H	FIC	Male	1	1	0	
E	Healthy	Female	1	1	0	
F	Healthy	Female	2	0	0	
I	Healthy	Female	2	0	0	
G	Healthy	Male	2	0	0	
J	Healthy	Male	1	1	0	
Total			16	4	0	

^a At least two of the three spectra from every film had to be correctly classified for the film to be considered identified.