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Research Article

Protein Profiling of Male Mouse Reproductive Tract Infected with Uropathogens

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ABSTRACT

Introduction: It is well acknowledged that bacterial uropathogens are a major factor influencing the male reproductive system. The present research aims to comprehend how uropathogens alter the protein composition of the testis and vas deferens when administered intratesticularly in male mice.

Materials and methods: The male mice were intratesticularly inoculated with a single dose of 20 µl (PBS) or 10^8 CFU/20µl (*Serratia marcescens, Escherichia coli, Staphylococcus aureus,* or *Klebsiella pneumoniae*). On Day 7, mice from each group were euthanized and the bacteria were reisolated from tissue homogenates of reproductive organs and reisolated bacteria were confirmed on selective agar media. Protein profiling of different reproductive organs was carried out via reverse phase HPLC analysis.

Results: HPLC chromatogram profile of reproductive organs when compared with control group showed that in testis, all four test groups inoculated with microorganisms recorded two peaks with marked decrease in the intensity as compared to control group, whereas, two additional peaks of retention time 4.487 min and 2.673 min were present in testis of mice administered with *Serratia marcescens* and *Klebsiella pneumoniae*, respectively. Several additional peaks in HPLC chromatogram were recorded in vas deferens tissue.

Conclusion: Infection with bacteria significantly altered the protein expression profile of the reproductive organs of infected mice. These differences provide light on the involvement of microorganisms in producing reproductive ailments, which may be due to certain microbial components and virulence factors. If found, these changed proteins in reproductive organs
might serve as helpful biomarkers for diagnosing infection.

INTRODUCTION

Male reproductive diseases have a significant clinical impact due to their link to life threatening outcomes such as higher vulnerability to STIs, infertility, and cancer. Therefore, early diagnosis is critical for avoiding underlying illnesses and their irreversible effects [1]. For this reason, there is a skyrocketing need for innovative diagnostic tools like protein biomarkers, which enable better and faster treatment of a variety of illnesses affecting the male reproductive system. Unfortunately, the existing laboratory tests still fail to explain the underlying mechanisms at a subcellular level that are associated with abnormalities of the male genital tract. Protein profiling data can provide us with better understanding and information that can aid in

improving and treating the reproductive tract disorders. For that purpose, investigating the differences between diseased and healthy state will help us to understand the pathology of male reproductive tract disorders and, eventually in near future, to treat them [2].

Proteins are postgenomic effectors of cellular and molecular events, protein expression profiling or the comprehensive quantification of proteins occurring in a fluid/ cell population has become a vital and recognized tool in understanding the mechanism of biological systems. Protein profiling has greatly contributed in the selection of distinctive proteins basedon their response to the particular conditions; either they are upregulated or downregulated or choosing the differentially expressed proteins and using them for the designing of new therapeutic drugs and reliable diagnosis. The defined and known group of proteins could be used for the identification of the unknown group of proteins and hence, making proteins suitable for the clinical diagnostics [3]. The microorganisms used in the present study are *S. aureus*, *S. marcescens*, *E. coli* and *K. pneumoniae*. *S. aureus* is commonly found in chronic cutaneous ulcers and is considered a serious pathogen. It causes a wide variety of skin and soft tissue infections [4]. *S. marcescens* is an opportunistic nosocomial pathogen which has been found to be associated with meningitis, endocarditis, septicaemia, respiratory infection and many other conditions [5].

E. coli causes urinary tract infections in people which can account for more than 85 percent of these illnesses. *E. coli* causes cystitis by entering the urinary tract via the urethra, and this infection may spread to the kidneys (causing Pyelonephritis) and ultimately cause kidney failure and sepsis if left untreated [6]. *K. pneumoniae* is one of the common bacteria that causes UTIs, pneumonia, and soft tissue infections. This infectious agent may colonise the digestive tract, spread to the rest of thebody through the circulation, and ultimately prove lethal [7]. Along with the above mentioned aetiologies, these uropathogens have been found to be associated with reduced male reproductive potential as well [8,9].

MATERIALS AND METHODS

Animals

Sexually mature, male BALB/c mice, 5–6 weeks old, 25 ± 2 g, were procured form the Central animal house of Department of Microbiology, Panjab University, Chandigarh, India. The animals were given standard pellet food consisting of 20-21% crude protein, 4% fat, 5.0- 7.5% crude fibre, 8-9% ash, 1.0-1.5% calcium, 0.6-0.8% phosphorus and 50% nitrogen free extract (M/s Ashirwad Industries Pvt. Ltd.) and water *ad libitum*. The prospective study procedures were approved by Institutional Animal Ethics Committee, Panjab University; Approval No. PU/45/99/CPCSEA/IAEC/2021/658 and the experiments were carried out ensuing the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Microorganisms and preparation of inoculum

The standard microorganisms viz. *Klebsiella pneumoniae* (MTCC 4030), *Serratia marcescens* (MTCC 7641), and two clinical isolates from semen of males viz. *Staphylococcus aureus* and *Escherichia coli* used in the present study were already present in our laboratory as glycerol stocks. Microorganisms were grown in Luria-Bertani broth (LB) under shaking conditions for 24h/ 37°C. The culture broths were centrifuged at 10,000 rpm for 10 min. The pellet so obtained was washed twice with Phosphate Buffer Saline (PBS) (50mM, pH 7.2). The final concentration of 10^8 cfu/20µl of each organism was achieved by suspending the pellet in PBS buffer. In order to assess the role of uropathogens in the reproductive potential of male mice, BALB/c mice (n=15) were divided into five groups (I, II, III, IV, and V):

1. Group I (n = 3): PBS
2. Group II (n = 3): 10^8 cfu of *S. marcescens* in 20µl PBS
3. Group III (n = 3): 10^8 cfu of *E. coli* in 20µl PBS
4. Group IV (n = 3): 10^8 cfu of *S. aureus* in 20µl PBS
5. Group V (n = 3): 10^8 cfu of *K. pneumoniae* in 20µl PBS

Intratesticular administration

Mice were anesthetized with ketamine (75mg/kg) and xylazine (121mg/kg) and under sterile conditions, the mice were placed in supine position and the whole abdominal area was thoroughly wiped with 80% ethanol. The abdominal area was gently pressed so as to protrude out the testis and was inoculated with the help of dispovan insulin syringe, a single dose of 20µl (uropathogen/ PBS).

Sample collection and processing

To follow the itinerary of experiment, mice from each group were euthanized on day 7. Right testes and vas deferens were removed and kept in separate sterile vials. With the use of the surgical blade and the hand homogenizer, the mincing and homogenization of the reproductive organs in PBS was done, so as to obtain 10% homogenate. The homogenized mixture was centrifuged at 10,000 rpm at 4°C to obtain cell debris free supernatant. The supernatant was collected into different sterile vials and further processed for confirmation of re-isolation studies and protein profiling.

Confirmation of reisolated microorganisms

The obtained bacterial isolates were streaked on Brain Heart Infusion Agar (BHI), Eosin Methylene Blue (EMB) agar, Mannitol salt agar (MSA), and MacConkey Agar for the confirmation of *S. marcescens, E. coli, S. aureus, K. pneumoniae*, respectively.

Protein profiling/chromatograms of tissue homogenates

Reverse-phase high performance liquid chromatography: The isocratic analysis was carried out on The UltiMate 3000 Basic Automated high performance liquid chromatography system. The system consists of 1-4 channels and compact automated column compartment with a quaternary pump. The UltiMate 3000 systems, combined with Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software is used for peak integration. A four-channel solvent degasser is integrated in the pump module to simplify operation (Thermo Fisher Scientific, Mumbai, USA). The mobile phase was a buffer prepared with 0.1 M sodium dihydrogen orthophosphate, 0.2 M Na_2EDTA. The pH of buffer was adjusted to 3.1 with orthophosphoric acid and acetonitrile (2.2-5% v/v). 0.22 µm syringe filter was used to filter the mobile phase. An ambient temperature was maintained for the operation of column and the constant flow rateof the mobile phase was 1 ml/min.

RESULTS

Re-isolation of administered microorganisms

S. marcescens was found to be Gram-negative, short and rod shaped. Further, for colony morphology, the obtained re-isolates were streaked on Brain Heart Infusion (BHI) agar plates. Testis and vas deferens confirmed the presence of *S. marcescens* by producing red colonies as depicted in Figure 1a-1d.

E. coli was found to be Gram-negative, rod shaped with rounded ends under the microscope. The test group confirmed the presence of *E. coli* by producing green metallic sheen in testis and vas deferens by streaking on Eosin-Methylene Blue (EMB) agar (Figure 1e-1h).

S. aureus was found to be Gram-positive, coccus shaped and appeared as grape like clusters under the microscope. The homogenates of all the reproductive organs of the test group were confirmed on Mannitol Salt agar (MSA) plates. The presence of yellow colonies with yellow zones only in the testes and vas deferens of test group confirmed the presence of *S. aureus* (Figure 1i-1l).

K. pneumoniae was found to be Gram-negative and rod shaped under the microscope. Presence of mucoid pink colonies in testes and vas deferens confirmed the presence of *K. pneumoniae* on MacConkey Agar (Figure 1m-1p).

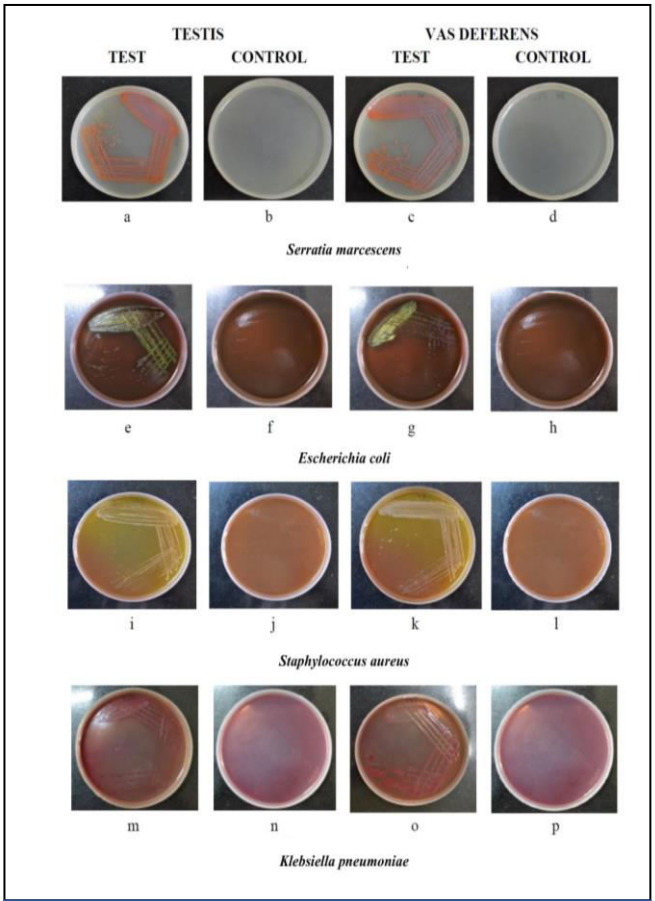

Figure 1: Representative photographs of Group I-V showing growth on selective agar plates on day 7; *Serratia marcescens* a-d, *Escherichia coli* e-h, *Staphylococcus aureus* i-l, *Klebsiella pneumoniae* m-p.

Protein profiling/chromatograms of tissue homogenates using RP-HPLC

Protein profiles of testes and vas deferens tissue homogenates from control and test groups were recorded using RP-HPLC. Each recorded chromatogram has a distinctive set of peaks. In testis of control group, five major peaks were detected with the retention time of 1.310 min, 2.220 min, 2.953 min, 3.530 min and 3.827 min at 254 nm (Figure 2a). Whereas, testis inoculated with *S. marcescens* showed a notable peak at a retention time of 4.487 min which was not detected in the control group. Also, a significant attenuation in the intensity of the two peaks at 2.967 min and 3.523 min were demonstrated (Figure 2b). This difference depicts a noticeable variation in the protein profile of control and *S. marcescens* infection induced testis tissue.

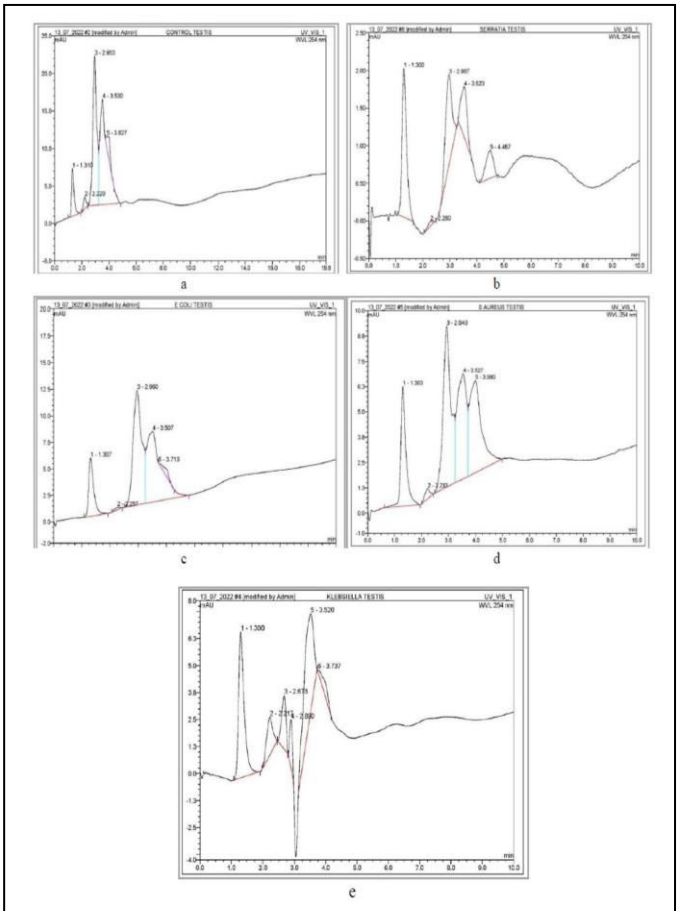

Figure 2: HPLC chromatogram profile of the testis intra-testicularly administered with (a) PBS, (b) *S. marcescens*, (c) *E. coli*, (d) *S. aureus*, (e) *K. pneumoniae* with their retention time and area at 254 nm.

In testis challenged with *E. coli*, a gradual shift in the peak was observed from 3.827 min in the control group to 3.713 min in the test group. The peaks at 2.960 min, 3.507 min were detected to be less intensified when compared to control group (Figure 2c). In the testis administered with *S. aureus*, difference in the peak at 3.980 min was apparent and two peaks with the retention time of 2.943 min and 3.527 min were found to be less intensified as compared to the control group (Figure 2d).Whereas, in *K. pneumoniae* inoculated test group, one new peak with retention time 2.673 min was observed and peaks at 2.890 min, 3.520 min
were found to be less intensified as compared to the control group (Figure 2e).

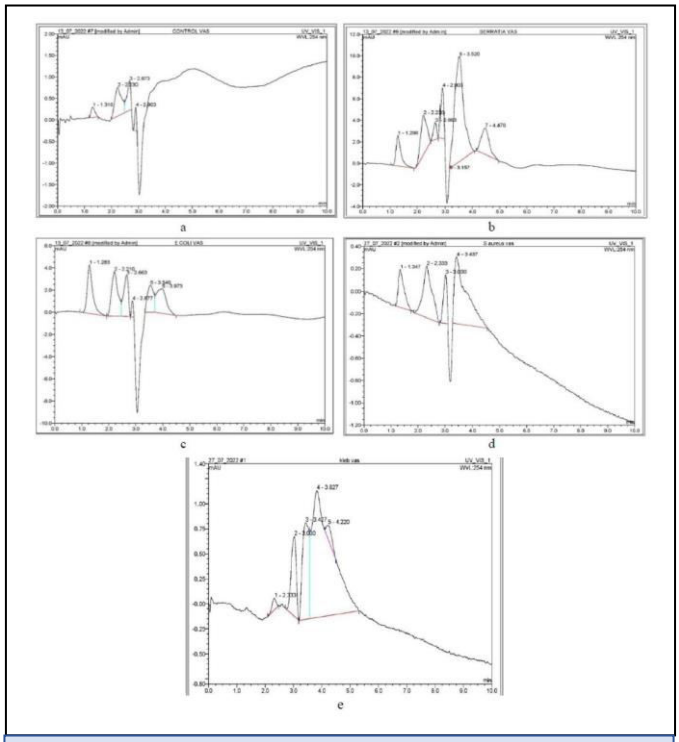

Figure 3: HPLC chromatogram profile of vas deferens tissue administered with (a) PBS, (b) *S. marcescens*, (c) *E. coli*, (d) *S. aureus*, (e) *K. pneumoniae* with their retentiontime and area at 254 nm.

Next, vas deferens protein profiling was performed. In control group, four major peaks were observed with the retention time of 1.310 min, 2.230 min, 2.673 min, and 2.903 min at 254 nm (Figure 3a). Whereas, vas deferens administered with *S. marcescens*, the peaks eluted at a retention time of 3.157 min, 3.520 min and 4.470 min were observed only in the test group that confirms the establishment of new proteins in the *S.*

marcescens infected state in the mice tissue (Figure 3b). Whereas, in vas deferens from mice administered with *E. coli*, new peaks eluted ata retention time of 3.570 min, and 3.973 min (Figure 3c) and in case of *S. aureus*, peaks with retention time of 1.347 min, 2.333 min, 3.030 min, 3.437 min 254 nm were detected (Figure 3d). The peaks 2.673 min, and 2.903 min observed in control group were lost in the test group. vas deferens from mice administered with *K. pneumoniae* depicted elution of new protein peaks at a retention time 3.030 min, 3.427 min, 3.827 min, and 4.220 min, which were all absent in the control group (Figure 3e).

DISCUSSION

To obtain a more comprehensive outlook on the protein profile of infected organs, High Performance Liquid Chromatography (HPLC) studies were performed. Several prior studies have demonstrated that the tissue protein profile using HPLC can be obtained for an early diagnosis with high specificity and sensitivity; such as, protein profile analysis of tissue homogenate in cervical cancer [10], oral cancer [11], breast tissue homogenates [12] and ovarian cancer [13]. In the current investigation, the protein profile chromatogram for testis and vas deferens tissue homogenates from control and test samples were recorded. Elution profile of control group were noted in the range of 1.31 – 3.83 min for testis. Notably, the findings highlight that when compared to the control group, two peaks with retention time of 2.9 min and 3.5 min were less intensified in the testis of all four-test groups (*S. marcescens, E. coli, S. aureus, K. pneumoniae*). Whereas, two additional peaks of retention time 4.487 min and 2.673 min were present only in case of mice administered with *S. marcescens* and *K. pneumoniae*, respectively. In vas deferens, chromatogram of control group was recorded in the range 1.31 – 2.90 min. Occurrence of 3 additional peaks in mice challenged with *S. marcescens* was determined within the range of 3.16 - 4.47 min. Whereas, *E. coli* exhibited 2 additional peaks with retention time 3.54 min and 3.97 min. Mice challenged with *S. aureus* also represented two new peaks with 3.03 min and 3.44 min. In mice inoculated with *K. pneumoniae*, occurrence of four extra peaks in HPLC chromatogram was recorded within range of 3.03 – 4.22 min.

Overall, these results are in substantial agreement with the previous findings where presence of new peaks by HPLC-LIF has been reported, such as Kodali et al. who examined the tissue homogenates of cervical cancers using normal and malignant cervical tissues [14]. The increased magnitude of the peaks in the malignant tissues suggested the alteration of protein quantitatively to certain extent. Also, Bhat et al [10] examined proteins that were present in elevated amount in the stage II samples of cervical cancer along with presence of many new proteins. HPLC-LIF detected 1861 and 1893 peaks in all the stages of the cancer as more intensified. Thus, each chromatogram for test group that has been recorded has its own unique collection of peaks. Each peak identified might represent a different protein, or, in certain cases, a mixture of proteins. An attempt to identify the specific proteins have been not made yet, as the purpose of our study was to test the hypothesis that proteins might be utilized for pattern- based disease detection.

Despite the fact that numerous peaks are shared by all five groups, the intensities of certain peaks differed significantly between the control and test chromatograms. Also, unlike the chromatograms of control group, emergence of additional peaks suggests the production of diverse proteins in the diseased condition that could be due to alteration of protein profiles during progression of bacterial infection in the tissues. These findings propose that bacterial infections induce changes in the protein profile of the infected organs, but each response is most likely influenced by the specific microbial factors. As during a bacterial infection, the levels of a number of reproductive organ proteins alter. The differences in an organism's protein profile may be linked to tissue types, internal and external environments, and developmental phases. According to a study by Alsubhi et al [15], protein profiles of samples from patients diagnosed with *E. coli*-induced UTI showed upregulation of proteins which can be further exploited as potential biomarkers. Albrethsen et al documented that majorly the downregulated proteins are involved in various information pathways, respiration, metabolism, whereas upregulated proteins are more widely distributed across virulence, adaptation and detoxification and cellular processes [16].

In the present study also, it is anticipated that alterations in the protein profile of the test mice's reproductive organs relative to the control mice may cause reproductive problems and

impotency. Although, in this preliminary study, altered expression of protein profile of the infected tissues has been observed, but a specific biomarker could not be elucidated. Further, these new proteins eluted in the present study, need to be identified.

CONCLUSION

Based on the above findings, it can be concluded that bacterial infection can lead to the alteration in the protein profiling of the reproductive organ such as testis and vas deferens of infected mice. These variations provide an insight into the role of the microorganisms in causing reproductive disorders that might be because of the specific microbial components and virulence factors. These alterations in the protein profile can be further analysed and extended for an early detection of genital tract infections and thus, may possibly act as a diagnostic biomarker.

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Host Institute

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interestassociated with this publication.

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