CLINICAL INVESTIGATION

Bacterial quality of urinary tract in patients with alkaptonuria

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ABSTRACT

Background: The aim of the current study was to determine whether there is an association between alkaptonuria (AKU) and urinary tract infection (UTI) by exploring the bacterial quality of the urinary tract, as most of the patients with AKU present with frequent occurrence of urinary tract symptoms such as incomplete emptying of urinary bladder, dysuria and nocturia.

Methods: Study samples were collected from 22 participants; 9 from patients with AKU, 9 from individuals who were AKU carriers, and 4 people served as control. Confirmation of AKU diagnosis was established by the ferric chloride test and quantitative determination of urinary homogentisic acid (HGA) levels.

Results: In the ferric chloride test, the urine samples of AKU patients showed a characteristic black ring upon addition of few drops of ferric chloride solution. During urinary HGA determination, patients with AKU had increased levels of urinary HGA as compared to carriers and controls. The following 10 bacterial species were isolated from the urinary tract of AKU patients, carriers and controls: *Sphingomonas paucimobilis, Escherichia coli, Francisella tularensis, Staphylococcus hominis, Staphylococcus haemolyticus, Leuconostoc mesenteroides, Dermacoccus nishinomiyaensis, Kytococcus sedentarius, Serratia fonticola* and *Granulicatella adiacens*. The presence of *S. paucimobilis* was found in three male patients, and one female each from the carrier and control groups. Almost all study samples were positive for *D. nishinomiyaensis* and *K. sedentarius. S. fonticola* and *G. adiacens* were found only in AKU carrier females.

Conclusions: The results deduced that males show symptoms of arthritis early and more severely than females and by this it appears that there is an association between these symptoms and the percentage of bacterial infection in males that requires more accurate diagnosis and treatment to clarify such relationship. In the current study, males (patients, carriers, and controls) were more likely to have bacterial infections than females (64% vs. 36%). The 16 and 2 bacterial isolates, detected in 7 males and 2 females AKU patients, respectively revealed that male AKU patients had a 2.3-fold greater rate of bacterial infection than female AKU patients. Therefore, further studies are warranted to investigate if there's any relationship between higher incidence of bacterial infections and development of AKU-related clinical symptoms in the male population.

Key Indexing Terms: Alkaptonuria; Urinary tract infection; Homogentisic acid; Arthritis. **[Am J Med Sci 2023;■(■):1–7.]**

INTRODUCTION

Alkaptonuria (AKU) (OMIM: 203500) also known as the "black urine disease", was first described by the British physician Sir Archibald Garrod in 1908, while he was illustrating the concept of inborn errors of metabolism.[1,2] It is a genetic disorder inherited in an autosomal recessive manner and caused by mutation in homogentisate 1,2-dioxygenase (HGD, EC.1.13.11.5) gene which maps to the human chromosome 3q21−q23.[3] This mutation leads to a deficiency in homogentisate 1,2-dioxygenase (HGD) activity which is involved in the catabolism of homogentisic acid (HGA), an intermediary product of amino acid (phenylalanine and tyrosine) metabolism.[4,5] This condition is characterized by accumulation of HGA in the body and the excess amount is excreted in the urine, imparting a distinct black color to the urine. The urine formed turns black upon exposure to air or alkali due to formation of a dark polymerized product.[6]

In the body, HGA undergoes oxidation and subsequent dimerization to form a melanin-like pigment that gets deposited in the connective and cartilaginous tissues throughout the body.[4] The deposition of this pigment in the tissues leads to ochronosis, the hallmark of AKU.[7] The molecular mechanism of ochronosis was recently elucidated using redox-proteomic analyses which provided a potential pharmacological basis for its treatment.[4,8,9,10,11,12]

AKU is a rare disease with a prevalence rate of approximately 1:250,000–1,000,000 in most ethnic groups. However, the incidence is higher in countries like Slovakia and the Dominican Republic where it is estimated to rise up to 1:19,000.[13] Recent studies have reported 40 cases of AKU in South Jordan.[14] However, the incidence of AKU in Jordan remains unknown. The features of ochronosis begin to appear usually around the third decade of life. Patients with AKU suffer from joint and spine arthritis and in more severe cases the cardiovascular system gets affected causing damage to the cardiac valves.[15] As the disease progresses, most patients develop renal stones; male patients are at an increased risk of developing prostate stones.[5] In a recent finding, a Jordanian male patient with AKU was admitted to the hospital for severe lower urinary tract symptoms (LUTS).[6] Urine analysis was normal and free of bacterial growth. Clinical examination, non-contrast urinary tract computed tomography (CT), and a Kidney, Ureter and Bladder (KUB) plain film revealed numerous stones in the bladder and prostate gland particularly deposited in the paraprostatic diverticulum.[6]

The current study aims to investigate if there is an association between AKU and the bacterial quality of the urinary tract (UT).

METHODS

Ethical consideration

This study was approved by the Institutional Ethics Committee (IEC) in The Deanship of Scientific/Academic Research & Quality Assurance at Mutah University. The study abided by the Declaration of Helsinki (DOH). Written informed consent was obtained from all participants.

Study sample

The target population was selected based on surveys, interviews, and review of medical history. Study samples were collected from affected individuals and carriers of AKU who complained of recurrent LUTS. No antibiotic therapy was administered for the last three weeks before sample collection. The control samples were collected from participants with no family history of the disease. A total of 22 individuals participated in the study. The study samples were obtained from 9 AKU patients (7 males and 2 females; ages 6–49 years), 9 AKU carriers (4 males and 5 females; ages 18–70 years), and 4 controls (2 males and 2 females; ages 20–56 years) (Table 1).

Diagnosis of AKU was based on clinical examinations and laboratory results. Urine samples were observed for change in colour from yellow to black upon standing, and quantitative measurement of urinary HGA levels using gas chromatography-mass spectrophotometry (GC-MS) was done. As expected, raised levels of urinary HGA were found in AKU patients. Additionally, loss of lordosis was detected by radiological examination (X-ray, MRI and CT-scan) of the spine, further confirming the diagnosis of AKU.

Urine culture

Sample collection

Fresh and early morning urine samples were collected from all the participants (patients, carriers and controls). To avoid contamination, the participants were requested to carefully clean their hands and external part of their genitalia with a disinfectant. Sterilized plastic containers were used for sample collection and the participants were informed to open them just before the sampling process. Mid-stream urine samples were collected and immediately transported to the laboratory in an icebox. The samples were cultured on a suitable media for 24-h under aseptic conditions and stored at 4 °C for further study.

Isolation, purification, and identification of bacterial strains

Under aseptic conditions, 50 ml of urine sample was filtered through cellulose nitrate membranes (0.45 μm pore size and 47 mm diameter, Sartorius). Each filter was then placed on the surface of nutrient agar (NA) plates and the cultured NA plates were incubated for 24-h at 37 °C (Incubator, J.P. Selecta, Spain). The growing bacterial strains were observed under a dissecting microscope (model SMZ, Nikon, Tokyo) and differentiated based on their colonial morphology. Each selected colony was then transferred to fresh NA plates for further purification. The purified bacterial strains were stored at 4 °C and each of these isolated bacterial strains were identified using the VITEK 2 microbial identification system (BioMérieux, France).

HGA concentration measurement

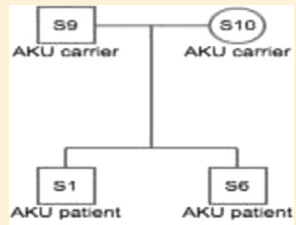
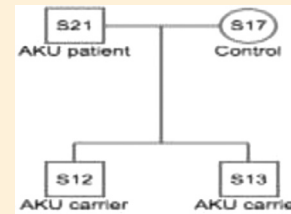
Sample collection

A 24-h urine collection was done from 8 AKU patients, 6 carriers and 4 controls. The urine samples were collected in 2.5 L plastic containers having 30 ml of 2 M H_2SO_4 as a preservative. After the sampling time of 24-h, the urine containers were agitated vertically in order to obtain a homogenous sample, then 40 ml was taken from each sample and stored in a separate sterile container. The samples were then transported to the laboratory and stored at 4 °C for quantification of HGA levels using GC-MS method.

Extraction and analysis

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is a widely used derivatisation agent in GC-MS analysis. The

Table 1. Homogentisic acid concentration in AKU patients, carriers and controls.

Sample ID		Participant medications	HGA concentration [mg/L]
AKU Patient male	S1	Vitamin C and Indomethacin (PRN)	31.84
	S2	Vitamin C, Indomethacin (PRN), and Levothyroxine	71.16
	S3	Selenium, Indomethacin (PRN)	78.04
	S4	NA	64.80
	S5	N.A**	69.00
	S6	N.A	55.99
	S21	Selenium and Levothyroxine	24.34
AKU Patient female	S7	Vitamin C and Levothyroxine	59.67
	S8	Selenium, Etoricoxib (PRN), and Levothyroxine	89.26
AKU Carrier male	S9	Indomethacin (PRN)	N.D*
		Vitamin D	
	S12	N.A	N.D
	S13	N.A	N.D
	S14	N.A	N.D
AKU Carrier female	S10	Multivitamin, Candesartan, Levothyroxine	N.D
	S11	N.A	N.D
	S15	Levothyroxine	N.D
	S16	N.A	N.D
	S22	N.A	N.D
Control female	S17	N.A	N.D
	S18	N.A	N.D
Control male	S19	N.A	N.D
	S20	N.A	N.D
Pedigree of Family 1 and Family 2			

* Not detected, ** Not available

derivatisation of HGA was performed according to Zafra et al.[16] with slight modification where a mixture of 20:5:25 (v/v/v) BSTFA-pyridine-ethyl acetate containing HGA was allowed to stand for 2 min at room temperature. A liquid urine sample of 1 ml was transferred into polypropylene test tube and 1 g NaCl, 200 μL of 5 M HCl and 6 ml ethyl acetate were added to the sample. The mixture was shaken well for 10 min then centrifuged at 4000 rpm for 3 min. The upper layer comprising of ethyl acetate was transferred into a vacuum test tube. The extracts were evaporated to dryness using a gentle stream of nitrogen. The residues were reconstituted in 750 μL of 6.65 mg/L internal standard (m-Methoxy-acetophenone) that dissolved in ethyl acetate using 250 μL of the previous solution mixed with 50 μL of pyridine and 200 μL of BSTFA. The mixture was shaken well for 2 min in order to derivatise the phenolic compounds, and then 1 μL of the prepared solution was injected into the GC-MS instrument.

RESULTS

The urine samples of affected individuals showed a characteristic black ring upon addition of a few drops of ferric chloride solution **(**Fig. 1**a, b and c)**. Another diagnostic tool used was quantitative determination of urinary HGA levels by GC-MS analysis **(**Table 1**)**. AKU patients had higher levels of urinary HGA when compared to carriers and controls. Further diagnosis was made on the basis of medical signs and symptoms. All the clinical manifestations observed in patients, carriers and controls were recorded **(**Table 2**)**. Apart from urinary tract infection (UTI), no other clinical manifestation was observed in carriers and controls.

Attempts were made to determine if any correlation existed between the bacterial species responsible for UTI in patients, carriers and controls. The current study did not show any dissimilarity in the bacterial species that were isolated from the urine samples of AKU

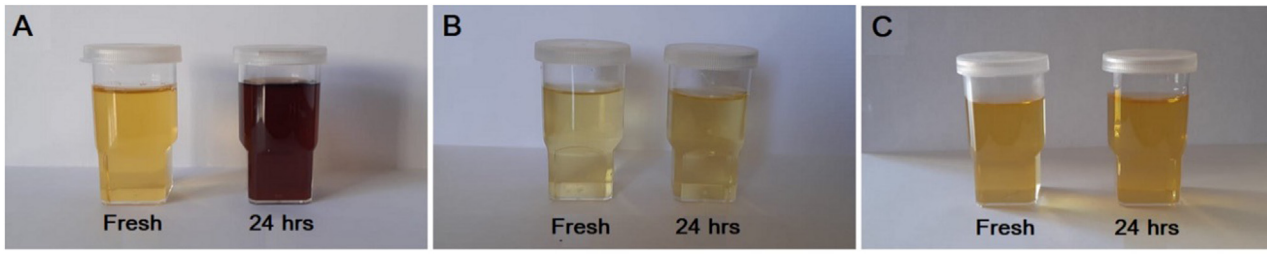


FIG. 1. Urine samples from AKU patient (A), AKU carrier (B), and control (C). Fresh urine appears normal yellow in all the samples and changes to dark black within 24-h only in the AKU sample due to presence of oxidized homogentisic acid. No color change in carrier and control samples.

patients, carriers and controls, irrespective of age and sex **(**Table 3**)**.

DISCUSSION

Urine samples from patients with a diagnosis of AKU turned black, whereas those collected from AKU carriers and controls did not show any color change **(**Fig. 1**a, b and c)**. Furthermore, AKU patients showed a clear rise in their urinary HGA levels as compared to carriers and controls **(**Table 1**)**. These results thus confirm the validity of the study samples.

It was noticed in the current study that 7 out of 9 AKU patients were suffering from morning stiffness, pain in weight-bearing joints such as knees and hips, and back pain due to multiple degenerative changes in the spine, and 6 out of 9 were suffering from discoloration of the ear pinna, sclera, and teeth caused by the deposition of ochronotic dark pigment. These findings were in accordance with several published studies.[17] One patient from the current study experienced a ruptured Achilles tendon as the disease progressed. Rupture of Achilles tendon is an important clinical manifestation of AKU and a few case reports have been published on it. It occurs due to accumulation of large amounts of HGA in connective tissues which subsequently weakens and ruptures the tendon.[18]

Renal and prostate stones were seen in 5 out of 9 AKU patients. The renal parenchyma is exposed to high concentrations of HGA as it is excreted from the kidneys exclusively by glomerular filtration and active tubular secretion, thus increasing the risk of renal stones in AKU patients (prostate stones in males). All AKU patients reported recurrent UTI symptoms, although their urine culture reports were negative. As a result, membrane filter technique was used in the current study to confirm if the urine samples were bacteria-free. Recurrent UTI was noted in 7 out of 9 of AKU carriers and 2 out of 4 of controls **(**Table 2**)**. In the current study, carriers were included in a group different from controls, although from genetic point of view carriers have a defective gene but do not show any AKU symptoms. Nevertheless, few carriers show mild symptoms depending on the type of food that they eat. For example, some carriers complained of brown staining in their clothes, particularly the underarm area, similar to AKU patients (direct contact with Jordanian AKU society). In unpublished data, AKU carriers showed tendency to develop hypothyroidism similar to AKU patients. Consequently, the carriers group was included as a separate group from the controls to see if there was any association or differences between the development of this genetic disease and bacterial quality of the UT as it has been found in their medical records, carriers complained of frequent UT symptoms similar to AKU patients.

Since many of AKU symptoms resemble urinary tract infections symptoms, it was necessary to examine the urine samples for their bacterial quality and quantity.[19] The urine analysis in many patients with AKU was normal and free from bacterial growth, but at the same time the clinical examination and the non-contrasting urinary tract and urinary tract (KUB) revealed the presence of many stones in the bladder and gland of the prostate particularly deposited in the para prostatic diverticulum.[6] Therefore, filter papers were the best option for determining whether urine samples were virtually bacteria-free.[20]

Table 2. Clinical manifestations and symptoms of AKU patients, carriers and controls included in the study.

Clinical signs and symptoms	AKU patients (*n*= 9) (7 M* and 2 F**)	AKU carriers (*n*= 9) (4 M and 5 F)	Control (*n*= 4) (2 M and 2 F)
Black urine	9	0	0
Discoloration of the teeth, ear pinna and/or sclera of the eye	6	0	0
Morning stiffness, knee, shoulder, hip and back pain	7	0	0
Rupture of Achilles tendon	1	0	0
Renal and/or prostate stones	5	0	0
Recurrent urinary tract symptoms	9	7	2

Table 3. Bacterial species isolated from AKU patients, carriers and controls.

Bacterial strain			*Sphingomonas paucimobilis*	*Francisella tularensis*	*Escherichia coli*	*Staphylococcus hominis*	*Staphylococcus epidermidis*	*Leuconostoc mesenteroides*	*Dermacoccus nishinomiyaensis*	*Kytococcus sedentarius*	*Staphylococcus haemolyticus*	*Serratia fonticola*	*Granulicatella adiacens*
Sample ID		**Age**											
AKU Patient male	S1	45	+					+					
	S2	18			+						+		
	S3	40			+				+	+	+		
	S4	11			+						+		
	S5	6	+	+			+		+	+			
	S6	44	+										
	S21	49											
AKU Carrier male	S9	70		+									
	S12	20			+								
	S13	22				+	+						
	S14	18						+					
AKU Patient female	S7	17			+								
	S8	50			+								
AKU Carrier female	S10	70			+								
	S11	44			+								+
	S15	38	+										
	S16	35							+	+		+	
	S22	40											
Control female	S17	42	+						+	+			
	S18	56							+	+			
Control male	S19	22			+				+	+			
	S20	20			+								

The urine samples of all the participants were analysed for bacterial composition. *S. paucimobilis* was detected in three male patients, and one female each from the carrier and control groups. The most common type of bacteria associated with UTI is *E. coli* which was found in all the samples.[21] It has special structures called fimbriae with which it adheres to the host epithelium. In the last two decades, molecular studies have identified 29 virulence factor genes in *E. coli*.[22] On the other hand, other types of pathogenic bacteria adhere to epithelial cells through various mechanisms like production of urease enzyme that stimulates urea degradation in the urine causing bladder or kidney stones.[23] *F. tularensis* and *L. mesenteroides* were found only in male patients and carriers. One male from the carrier group was detected with *S. hominis*, while *S. haemolyticus* was observed in males from the patient group. UTIs were rated as one of the most common intercontinental infections, with higher risk in male than female patients by 2.2-fold who suffered acute appendicitis.[24]

D. nishinomiyaensis and *K. sedentarius* were found in almost all study samples. *S. fonticola* and *G. adiacens* were found only in females from the carrier group. It was reported that while the acute appendicitis inflammatory of fluid is occurring, then its bacterial contents could flee to the blood flow and peritoneal chamber, thus transmission of pathogens to the subaltern locations.[24] Males (patients, carriers, and controls) in the current study had a percentage of bacterial infections as high as 64%, compared to females' percentage of 36%. Additionally, there were 16 and 2 bacterial isolates in 7 males and 2 female AKU patients, respectively, indicating that male AKU patients had a 2.3-fold greater rate of bacterial infection than female AKU patients.

CONCLUSIONS

Our study could not establish any association between AKU and the type of bacterial species inhabiting the UT despite the fact that most AKU patients reported recurrent UT symptoms. The UT symptoms like incomplete emptying of urinary bladder, dysuria and nocturia might be secondary to obstruction and irritation caused by HGA as it passes through the kidneys during its removal. The incidence of AKU is equal in males and females. However, arthritic symptoms occur earlier and with a greater degree of severity in males than females.[25] Bacterial presence turned out to be 64% in males (patients, carriers and controls), whereas, it was only 36% in their female counterparts. Additionally, the male AKU patients had a 2.3-fold greater rate of bacterial infection than female AKU patients. Further studies are warranted to investigate if there's any relationship between higher incidence of bacterial infections and development of AKU-related clinical symptoms in male population.

ETHICAL CONSIDERATION

The study abided by the Declaration of Helsinki (DOH). All ethical principles for medical research involving human subjects were enforced. The human subjects' confidentiality and rights were preserved throughout the study.

AUTHORS CONTRIBUTIONS

Amjad and Muhamad recruited the patients and collected the samples. Amjad, Ali and Ibrahim analysed the samples. Nesrin, Khaled and Hussam designed the experiments and screened the relevant literature review. Nesrin, Amjad, Moath and Hussam revised and interpreted the patients' medical records and drafted the manuscript. All authors have read and approved the final manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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