Comparative analysis of microbiome of bladder cancer patients

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Abstract

Background: It has been studied that the urinary tract, which was once considered to be sterile retains a unique microbiome. The current study was performed to explore the microbiome of male and female cancerous bladder tissue, including 01 control sample and 09 cancer samples using 16S rRNA gene sequencing. In previous studies, the significance of microbiome has been found to be associated with the bladder cancer. For diversity analysis, V3-V4 regions of 16S rRNA were used for PCR and later sequencing was carried out through Illumina, Miseq platform. The metadata generated was analyzed on QIIME 1.9.1.

Result: The bacterial diversity detected showed that five of the phyla; namely *Proteobacteria* (38.1%), *Firmicutes* (37.8%), *Actinobacteria* (5.9%), *Thermi* (4.9%) and *Tenericutes* (2.5%) were more abundant in all samples as compared to other phyla. The genera found in all samples were *Enterobacter* (18.3%), *Bacillus* (13.9%), *Meiothermus* (4.9%), *Methylotenera* (1.2%), *Ralstonia* (3.6%) and *Streptococococcus* (1.4%). *Ralstonia* and *Streptococcus* were absent in BLC 10 and BLC 2, respectively, while present in the rest. The results of alpha and beta diversity showed that female samples had more bacterial diversity and uniformity as compared to male samples.

Conclusion: The present study used biopsy samples of newly diagnosed cancer patients without taking into account any treatment given to the cancer patients. Our analysis showed insignificant differences in alpha and beta diversity of male and female samples. The genus *Meiothermus* detected in this study was firstly reported in a bladder microbiome analysis. The data generated from such preliminary futuristic study can help in devising new diagnostic tools and therapies.

Background

The advancement in 16S rRNA analysis through the development of latest bioinformatics tools enriches the knowledge regarding microbiome of a particular community such as soil, water and even humans [1-4]. The idea to study the human microbiome came into existence when gaps were found to be present during the working on human genome project. Therefore, Human Microbiome Project (HMP) started with the purpose to fill the remaining gaps by studying and accessing the microbial diversity of five human body sites: skin, the gastrointestinal tract, mouth, vagina and the nasal cavity [5,6]. The microbiome of humans is said to be called the hidden organ as findings of the HMP refers that the number of microbes inhabiting the body of humans is much higher than body's own cells [7]. They does not only inhabit the body of humans but also play a vital role in different body functions such as maintaining health, involvement in the immune system, response to certain diseases while also providing a barrier against pathogens [8,9].

The gut microbiome is extensively studied one because gut microbiome has been found to play a central role in maintaining homeostasis of the body. The major abnormalities occur in our brain, heart, musculoskeletal and metabolic process occurs as a result of GI dysbiosis [10,11,9,12]. For example, the *Firmicutes* were found to present in larger numbers in obese individuals than lean while *Bacteroidetes*

number plummeted [13,14]. This shows that how the microbiome varies in different situations and possesses a potential to be used as a biomarker [12, 15, 1]. The last few years have confirmed the presence of microbes in the bladder too, while rejecting the long held belief of urinary bladder sterility [16,17]. The explored microbiome of urinary tract had shown that it possesses the microbes that were previously absent in culture dependent methods, and are distinct from gut microbes as well [18,19]. The number of studies was formerly conducted on both male and female samples of different ages and pathological condition by using urine samples, “clean-catch, urethral swab sample and midstream voided urine samples” in order to study bladder microbiome [20-22]. The outcomes revealed the difference among accessed microbiome based on sex, diseased and overall health condition of subjects [23, 22, 20,24]. Lately, published report carrying the information of explored microbiome of healthy and cancer male participants has confirmed the existence of unique and distinctive bladder microbiome of both healthy and diseased samples [25-27].

Bladder cancer is the 9th most commonly occurring types of cancer worldwide. The males are more prone to have it than females with ration 10:3 [28, 29]. According to WHO 2012 report, it is found to be 12th most commonly occurring type of cancer in Pakistan [30]. It has been noted that the disease relapses after treatment [31]. Hence, it is very important to find a way which somehow delays the recurrence of bladder cancer or completely eliminates the disease from the body.

The present study was conducted as a pilot project to access the microbiome of bladder cancer patients through biopsy samples as suggested by Wolfe et al., 2012 [32] that biopsy sample would be better quality material to represent the actual bladder microbiome. The current study included heterosexual samples: 6 males (one control male sample) and 4 female samples making total of 10. The main purpose of this study was to explore the actual microbiome of newly diagnosed cancer patients without taking any chemotherapy.

Results

Exploration of microbial diversity of human bladder through QIIME

The total number of sequences of 16S rRNA gene obtained from all samples was 769126. Out of the sequences, 718,363 sequences were identified as bacteria, 796 sequences as archaea and 49,993 sequences related to unclassified bacteria or archaea. A total 13 phyla, 22 classes and 84 genera were observed in total 9 samples. The main phylum that was found to be present in both male and female samples was *Proteobacteria* (2.5-82.2%). Other dominant phylum *Firmicutes* ranged between (5.3-80.7%). It was interesting to note that the only control sample showed the highest percentage of *Proteobacteria* (63.9%) in comparison to *Firmicutes* which was 9.6% in the control sample (Fig. 1). Also, female samples had shown the highest bacterial diversity as compared to male samples.

Heatmap was also generated at the phylum level study. The map represented (Fig. 2) the bacterial diversity in terms of colors. It was observed that the darkest color intensity showed at zero on scale by

default, the next darkest color on a scale represented the maximum bacterial diversity. From this point onward, the decrease in color intensity was shown a decrease in bacterial diversity in the samples at the phylum level. Collectively, the maximum bacterial diversity was seen in BLC3 and BLC4 (Fig. 2) while BLS1 showed minimum diversity. Based on gender, it was observed that the unique classes of archaea, such as class Miscellaneous Crenarchaeotal Group (MCG), and *Thermoplasmata* were found only in female sample BLC4 (0.1%). While *Methanomicrobia* found in only one male sample BLC3 (0.1%). However, the classes found in abundance were shown in a Table 1.

Bladder Microbiome Analysis at Genus Level

The sequences were identified by assigning taxonomy to observed OTUs counts by using RDP classifier within QIIME. The sequences classified mainly as unassigned, unclassified_*Enterobacteriaceae, Bacillus, Meiothermus* and *Methylotenera* were most copious among the rest. The *Streptococcus* (absent in BLC2 only), *Paracoccus* (absent in BLC7), other_*Enterobacteriaceae*, *Rolstonia* and *Acinetobacter* (absent in BLC10), *Corynebacterium* (absent in BLC2 and 4), unclassified_*Cytophagaceae*, unclassified_*Comamonadaceae*, unclassified_*Bacillaceae*, *Staphylococcus* and unclassified_*Streptophyta* (absent in BLC6 and 10), while *Pseudomonas* and *Burkholderia* (absent in BLC6, 7 and 10 only) were also prominent one. Conclusively, an area density graph and bar chart (Fig. 3 and Fig. 4) showed that BLC3 and BLC4 were the most diverse samples as compared to the rest while BLC6 and 10 were the ones with lowest diversity.

Alpha and Beta Diversity Analysis

The rarefaction curves observed OTUs and Chao1 were made on the gender basis have shown less OTUs in males as compared to female samples (Figs. 5). A similar trend was studied in observed OTUs, where female samples showed more diversity and uniformity than male samples. Moreover, T-test was performed for alpha diversity plots based on the gender division of samples; the P value was 0.5179 for Chao1 and 0.5521 for observed OTUs. The same test was run for control and diseased division of samples that presented the P value 0.8175 (Figs. 5). The output of command ran on QIIME for ANOSIM with 44028 permutation provided P value as p=0.8889 for sample IDs for beta diversity plot (Fig. 6).

Discussion

The current study sheds a light on microbiome of male and female cancer patients. The study conducted through biopsy samples suggested by Wolfe et al., 2012 was to present the actual picture of the microbiome. Therefore, 10 (BLC8- female sample excluded, since it failed to meet set criteria of sequencing) heterogeneous subjects were enrolled, including one control male sample. Accordingly, in data analysis, there were no statistically prominent differences observed in alpha and beta diversity compared on the basis of gender division in the samples. However, female samples have shown more number of OTUs as compared to male samples.

Presently, the composition of cancer patient's bladder microbiome is studied through biopsy samples. While, previous studies have been conducted using urine ("clean-catch, urethral swab sample and midstream voided urine samples") samples of healthy versus diseased heterogeneous population [20,21,22, 27]. Investigation on the urinary microbiome of subjects with different urological conditions such as urgency urinary incontinence, neurogenic bladder dysfunction, sexually transmitted infections had a high variation between samples based on their gender, health and different medical conditions [22, 33, 34, 35]. Similarly, studies on urinary microbiome have helped in better understanding of its association with diseases and it has been reported to have helped in the cure of diseases as well. For example, the recurrence of bladder cancer can be delayed with the help of *Lactobacillus casei* together with epirubicin, by reducing chronic inflammation [36]. In concordance of such finding the present study was designed to detect the microbiome of heterogeneous populations with bladder cancer.

Urothelial bladder cancer is the major type of the urinary tract malignancy which accounts for 145,000 deaths annually. Smoking is the main cause of bladder cancer. Additionally, a diet rich in fat has also found to increase the chances of developing bladder cancer [37]. The microbiome association and incidence of cancer has not clearly been known at present, but a recent report on bladder cancer has highlighted microbiome of cancer patients [27].

In current study, the main phyla observed were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria [27]*, *Actinobacteria, TM7 and Tenericutes* which have been confirmed by earlier studies as well [20,21,38]. Control sample (BLS1) and the rest of the diseased samples showed the marked differences at phylum level, e.g. in all cancer patients, except BLC5, phylum *Firmicutes* was found to be dominant than *Proteobacteria* with exception in BLC5 (youngest patient). Since the only control sample is not enough to draw significant conclusions yet the data for diseased sample suggested that it might be a possibility that *Firmicutes* are increased in cancer state [38, 27]. It has been reported that in female samples, the abundance of *Actinobacteria* and *Bacteroidetes* was higher than males, the same trend observed in the current study with a few variations [20,21]. In general, "intra individual variations" were detected at the phylum level studies [20] which have been demonstrated in previous studies as well. This poses a problem in defining the core microbiome of the bladder.

The occurrence of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacilli* and *Clostridia* bacterial classes mentioned here have been reported previously with variable quantity based on overall health or disease state of subjects studied [22,39]. Moreover, the presence of order *Clostridiales* has been attributed to healthy vaginal flora where they are of clinical significance for their use in the diagnosis of bacterial vaginosis [40].

Bacilli were more abundant in tumor samples, while *Clostridia* in non-tumor samples [38], and with the same correlation were observed in the current study. Of note, it was observed that the *Pseudomonas* or *Anerococcus* was found to be more abundant in samples where *Streptococcus* is low and vice versa. The same trend was observed in the current study wherein female sample BLC6, *Anerococcus* have shown a maximum abundance (19.6%) while *Streptococcus* showed the lowest abundance (0.1%), and inverse

case was observed in male sample BLC3 in which *Anerococcus* was 0.2 % and *Streptococcus* was 6.1% [39]. In various studies, the presence of *Streptococcus* associated with commensal flora of healthy males [23, 33]. However, it has found in cancerous and non-cancerous samples in the present study (except BLC2) and Xu et al [39]. It was suggested that the cancer might have a role to play in altering the composition of urinary microbiome [39]. Moreover, *Anaerococcus* has shown an unusual pattern of occurrence in male samples as it was absent in most of the males in this study while in BLC 10 (18.8%) its abundance is next to BLC6 female sample.

In our study *Lactobacillus* was found to be present in all samples, except BLC6 and 10, ranging between 0.1- 3.3%. The presence of *Lactobacillus* has been reported in earlier studies carried on healthy and one undergo urogynecological surgery females, and in a male study [41, 21]; these studies have confirmed the abundance of *Lactobacillus* more in healthy female than males [42, 43, 17]. Also, the abundance of *Lactobacillus* was found to decrease in disease state like in patients with interstitial cystitis as compared to healthy subjects [21,33]. In this study the *Lactobacillus* was found to be more abundant in two females than male, but was absent in two diseased samples including one female (BLC6 and BLC10).

Staphylococcus has been reported in previous studies conducted on both females (with and without overactive bladder) [42] and male (with a sexually transmitted infection) [21]. In current finding *Staphylococcus* ranged between 0.1-2.4% in all subjects except BLC6 and 10. *Peptostreptococcus*, commonly found in human commensal flora has shown the highest abundance in BLC10 (22.2%) while zero abundance in the control sample. The same trend has been reported in a former study [38]. Another study conducted on a heterogeneous population confirmed its presence in females between 50-69 years of age, while in the case of males, it was found to be present at age 70 or above [22]. The same trend was observed for females in the present study: BLC6 (21.4%) abundance observed, followed by BLC4 (0.1%) while BLC5 the youngest female has shown zero abundance for *Peptostreptococcus*. On the other side, in male samples maximum enrichment of 22.2% found in BLC10 (53 years) and 0.1% in BLC3 (50 years) which is not in accordance with Lewis (2013) studies except sample BLC2 (0.1%) of 72 years [22].

In the present study sequences belonging to different genera: unclassified_*Enterobacteria, Acinetobacter*, *Methylotenera*, *Rolstonia*, *Paracoccus*, *Burkholderia*, other_*Enterobacteriaceae*, *Pseudomonas* and unclassified_*Methylobacteriaceae*. The presence of these genera has been reported in previous studies in bladder through different sampling procedures [32, 42, 39].

A recent report in Nature Journal, *Fusobacterium*, *Actinobaculum*, *Facklamia* and *Campylobacer* were abundant in male samples while *Veillonella*, *Streptococcus* and *Corynebacterium* in healthy samples. Conversely, in this study, these genera were absent, only *Fusobacterium* was found in two male samples (BLC3 and 9) [27] whereas only *Corynebacterium* detected in control sample among the rest of genera mentioned in a report of Nature. Currently, the sequences belonging to *Acinetobacter* were detected in all samples (except BLC6 and 10) ranging from 0.3 to 22.6%. The *Acinetobacter* is declared to be a most abundant genus in healthy and urothelial carcinoma subjects in a previous study [39]. The sequences

belonging to unclassified_*Enterobacteriaceae* present in all samples have also been reported by another study on a heterogeneous population (Order: *Enterobacteriales*) as the most abundant taxa [33].

Paracoccus in the present study was detected in all subjects except BLC7, ranging as 0.1-3.6%. Its presence has been reported in the human skin microbiome [44]. *Pseudomonas* was detected in our study in all samples except these: BLC6, 7 and 10. Its presence has been reported in an urothelial carcinoma study earlier [39] and in overactive bladder female samples [42]. *Methylotenera* detected in all samples in the present study ranged between 0.1- 3.3% had been previously detected in a study on peritoneal tumors in humans along with multiple other genera [45].

The sequences belonging to genus *Ralstonia* were found in all samples except BLC10, ranging from 0.1-13.9%. *Rolstonia* are categorized as human opportunistic pathogens that play vital role in degradation of xenobiotics and recalcitrant compounds [46]. The occurrence of *Ralstonia* in the bladder has been reported previously [32, 21]. Presently, *Burkholderia* was detected in all samples except in BLC6, 7 and 10, ranging between 0.1- 1%. The presence of *Burkholderia* in the current study is in accordance with previous study where it occurred as a low abundant genus [32].

The sequences belonging to genus *Meiothermus* were detected in all samples ranging from 0.3-22%. The member of this genus was previously placed in *Thermus*. In 1996, new genus named *Meiothermus* was introduced. They belong from Gram-negative aerobic microbes that are able to make short filaments and normally found in thermal vents [47, 48]. It is noteworthy that *Meiothermus* has not been previously reported in the urine sample, but it was detected in all samples in the present study.

Additionally, *Ureaplasma* were found to be present in BLC6 (11.8%) and BLC10 (10.9%) only and other_*Bacillaceae* were detected in a range of 0.1-1.1% in all samples except BLC10. While *Lysobacter* were found to be present in all female samples and a male sample (BLC9), ranging from 0.1-0.5%. The presence of anaerobic and uncultivated fastidious bacteria, such as *Ureaplasma* has been confirmed in different studies on both, male and female bladder studies [25,21,43,32]. The presence of genus *Corynebacterium* found in the present study was low, ranging from 0.1-1.3%. Its presence has been reported in healthy male samples in different studies, confirming the current findings, e.g., high range (1.3%) was observed in the male control sample (BLS1). Previous findings suggested that *Corynebacterium* is expected to have a role in “the healthy urine microbiome” [21, 33].

In our study, the OTUs observed in both sexes through non-parametric estimation used to make rarefaction curves have shown more richness and evenness of bacterial diversity in females as compared to males (Figs. 5). There is no statistically significant differences were observed in the microbial profile of both sexes in current study, which is in concordance with previous studies [22]. Overall, beta diversity calculations showed insignificant differences. However, based on clustering together, it was observed that BLS1 (control sample) and BLC5, and BLC3 and BLC4 were clustered with each other and showed similarity among themselves. Likewise, BLC6 and BLC10 have depicted close proximity and same number of genera as well. While BLC2, BLC7 and BLC9 were not clustered with any other sample and represents dissimilarity with the rest of the samples. Variations between samples on the basis of gender have shown

that BLC2 and BLC7 have marked variation with female samples as they were not clustered with any female sample.

Conclusion And Perspectives

To our knowledge, the present study is the first metagenomic study of males and females with bladder cancer performed in Pakistan. The novelty of this study lies in its sampling material as till date it is the only study of urinary bladder cancer conducted through biopsy sample. Briefly, at phylum level control sample and diseased samples showed differences, however, one of the diseased sample (BLC5) showed noticeable similarity with a control sample. Likewise, male sample (BLC10) and female sample (BLC6) showed prominent similarity at genus level. Moreover, alpha and beta diversity analysis showed the statistically insignificant difference between male and female samples, and a control sample. Interestingly, the genus *Meiothermus* detected in this study has not been reported earlier in bladder microbiome and tumor studies. The data generated from such preliminary futuristic study can help in the newly emerging area like phenomics and the sustainable development goal revolution worldwide. The 'good health and well-being' can be supported through microbiome studies by devising new diagnostics and treatments against fatal diseases such as bladder cancer. However, the current study considers bacterial and archaea profile only, but along with these other microbes like viruses, fungus and protozoa also need to be considered as suggested by Jennifer Wargo (2019) [1].

Methodology

Samples collection and history

The biopsy samples of cancer patients were collected from Urology department, Jinnah Hospital Lahore, Pakistan. The surgery was performed by experienced medical professionals. The samples were collected based on specific criteria. From October 2015 to February 2016, ten (10) adults with bladder cancer were enrolled in this study. The Inclusion criteria were patients aged ≥18 years who were diagnosed with cancer. Exclusion criteria were; patients seeking chemotherapy or radiotherapy, UTI, use of any vaccination within past last years and age ≤ 18 years details are mentioned in Table 2.

The exclusion and inclusion criteria were addressed by the questionnaire and medical history was verified before surgery by considering the medical reports (urine completely and blood reports). In this study, 6 males and 4 females were enrolled. BLS1 is the biopsy sample that showed no bladder growth during surgery, however, pre-operative symptoms were of cancer was preceded as a control sample. Prior to participation, all subjects were informed and were asked for consent. The form was approved by the Ethical Review Committee of Forman Christian College to avoid any ethical constraints.

Metagenomic DNA isolation

Metagenomic DNA was isolated from bladder biopsies of cancer patients through Fast DNA spin kit Fast Prep ® kit (MP Biomedicals, USA), using the manufacturer's instruction. Briefly, 1g of biopsy samples

were thawed (preserved at -80°C in saline) and transferred in an autoclaved pestle and mortar for crushing with liquid nitrogen. Later user manual protocol followed. The concentration of Metagenomic DNA was checked qualitatively on 0.8% (w/v) agarose gel at 70-80 V for 45 min, and quantitatively on Nanodrop (Thermo Scientific NanoDrop 2000/2000c Thermo Scientific, USA) at 360 nm which ranged between 38.7 ng/ µL - 518.4 ng/ µL. Collectively, 10 samples were taken, including 4 female and 6 males samples. DNA was stored at 4 °C until 16S rRNA gene amplification.

Amplification of 16S rRNA and library validation

The amplification was done by using primers 341F (5' CCTACGGGNGGCWGCAG -3') and 805 R (5'-GACTACHVGGGTATCTAATCC- 3') for V3-V4 region of 16S rRNA gene, linked to specific linker and adapter sequences. The details of PCR conditions were as a dual index approach performed by Fadrosh et al. 2014 [49]. Shortly, the amplification of pure DNA was carried out by using reaction mixture of total 25 µL volume in which, 2x 12.5 µL PCR master mix (Greentech) 2 µL DNA template, 1 µL of each V3-V4 region specific primers were added and the total volume was raised up to 25 µL by adding nucleases free water. The PCR conditions were pre-denaturation for 5min at 95 °C, followed by denaturation of 30 cycles at 95 °C for 40 sec, annealing at 55 °C for 40 sec while extension and final extension at 72 °C for 1 min and 5 min, respectively. The amplified PCR products were purified with the QIAquick PCR purification kit (QIAGEN, USA) and pooled in equimolar concentration.

Sequencing of 16S rRNA gene through Illumina MiSeq

The MiSeq (Illumina Inc., USA) was used to perform at ChunLab, Korea. The dual-index approach was followed for the sequencing of 16S rRNA gene amplicons in order to avoid “low quality diversity reads”. The dual index approach has an advantage that it gives high quality reads [49]. Good quality fastq files were generated by using PrinSeq (v 0.20.4) and then the paired files were generated by using the ‘pear paired-end read merger’ tool on Galaxy. The further steps were carried by using default parameters in QIIME (1.9.1). *De novo* OTU picking was done to generate OTU files using UCLUST that is a default parameter of QIIME [50], with 97% sequence similarity and RDP classifier was used to assign taxonomy [51]. The files generated as an output of working on QIIME included bar charts and area charts of bacteria diversity classification of phylum, class, order, family and genus level. In addition, heatmap was generated using phylum level data along with production of alpha and beta diversity plots. Alpha and beta diversity were calculated using multiple_rarefactions.py and beta_diversity.py, respectively. The phylogeny based unweighted UniFrac distance matrix was used to find a difference between male and female samples. The rarefaction curves Chao1 and observed OTUs were made on the gender basis and control and diseased samples as well. The principal coordinate analysis (PCoA) and 3D plot for unweighted UniFrac distances were constructed to evaluate microbiome variation between samples [52]. T-test and ANOSIM were performed to calculate the significant differences between male and female samples.

Abbreviations

WHO: World Health Organization; GI: gastrointestinal tract; UTI: urinary tract infection; MCG: Miscellaneous Crenarchaeotic Group; OTU: Operational Taxonomical Unit.

Declarations

AVAILABILITY OF DATA MATERIALS

Sequence Read Archive analyzed through QIIME (1.9.1) in the current study is available at National Center for Biotechnology Information: (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA494288) under accession numbers SAMN10160244 to SAMN10160252.

Ethical approval and consent to participate

The current study approved by the Ethical Review Committee of Forman Christian College (A Chartered University) and Jinnah Hospital, study number ERC-13-2016.

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Author Contributions

AI: Conducted experiment, analyzed data and prepared manuscript; SZ and SM: help in data analysis and manuscript; KA: edited manuscript; SA: provided biopsy samples and approved final draft; MI: supervised research and reviewed the manuscript and KAM: supervised research and helped in study design.

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Competing interests

The authors have declared that they have no competing interests in the publication.

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Tables

Table 1 Microbial classes placed in hierarchical order identified in all subjects

Classes	Total %	BLS1	BLC2	BLC3	BLC4	BLC5	BLC6	BLC7	BLC9	BLC10
Gammaproteobacteria	**29.2**	48.3	59.5	17.5	12.9	75.8	1.9	10.9	34.6	1.9
Bacilli	**24.8**	8.6	24.2	31.6	36.8	5.0	6.3	80.7	23.8	6.3
Clostridia	**12.9**	0.9	0.2	3.1	2.7	0.3	54.2	0.0	0.3	54.2
Unassigned	**6.5**	26.3	4.8	3.4	2.7	6.2	3.6	4.7	2.1	4.8
Actinobacteria	**5.9**	48.3	59.5	17.5	12.9	75.8	1.9	10.9	34.6	1.9
Betaproteobacteria	**5.9**	4.3	2.6	15.8	16.2	5.9	0.3	1.0	6.6	0.2
Deinococci	**4.9**	5.7	5.6	3.7	1.3	3.6	0.4	1.7	22.0	0.3
Alphaproteobacteria	**2.8**	0.7	1.8	7.4	10.0	0.5	0.5	0.2	4.0	0.4
Mollicutes	**2.5**	0.0	0.0	0.0	0.0	0.0	11.8	0.0	0.0	10.9
ML635J-21	**1.2**	0.8	0.3	3.6	4.9	0.9	0.0	0.0	0.8	0.0
Chloroplast	**0.8**	0.2	1.1	3.0	2.6	0.3	0.0	0.1	0.5	0.0
Bacteroidia	**0.5**	1.1	1.5	1.0	1.0	0.1	0.0	0.0	0.6	0.0
Cytophagia	**0.4**	0.4	0.2	0.7	0.6	0.4	0.0	0.1	0.9	0.0

Table 2 Patients' History: gender, age and medical condition

Patient's code	Sex	Age	Medical Condition
(BLS1) Control	Male	73 years	Symptoms were similar to cancer, but there was no growth observed during cystoscopy.
(BLC 2) Diseased	Male	72 years	Newly diagnosed with Bladder cancer
(BLC 3) Diseased	Male	50 years	Newly diagnosed with bladder cancer Smoker 3-4 years
(BLC 4) Diseased	Female	40 years	Newly diagnosed with bladder cancer UTI- *E.coli* (menupause)
(BLC 5) Diseased	Female	32 years	Newly diagnosed with bladder cancer Diabetic – glucophage 500g Hypertensive
(BLC 6) Diseased	Female	61 years	Newly diagnosed with bladder cancer Non-smoker
(BLC 7) Diseased	Male	65 years	Newly diagnosed with bladder cancer Childhood smoker
(BLC 8) Diseased	Male	70 years	Newly diagnosed with bladder cancer Blood pressure- 1 year back
(BLC 9) Diseased	Male	63 years	Newly diagnosed with bladder cancer
(BLC 10) Diseased	Male	53 years	Newly diagnosed with bladder cancer Smoker for last 20 years

Figures

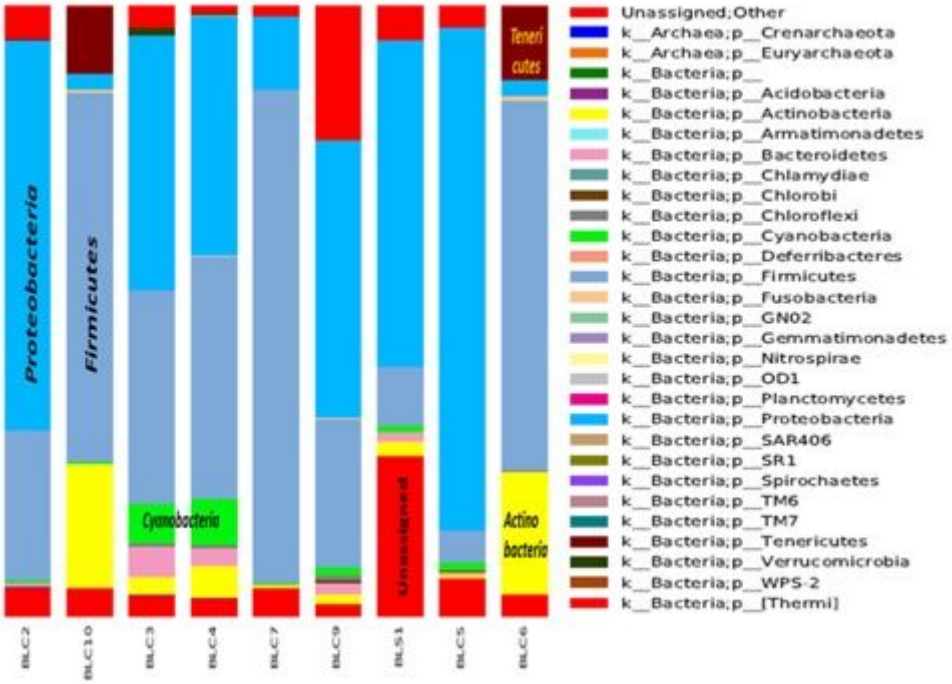

Figure 1

The bar chart showed the number of phyla found to be present in human bladder of females and male samples. Briefly, the only male control sample and one female diseased sample were found to be dominated the Proteobacteria while rest male and female diseased samples dominated by Firmicutes.

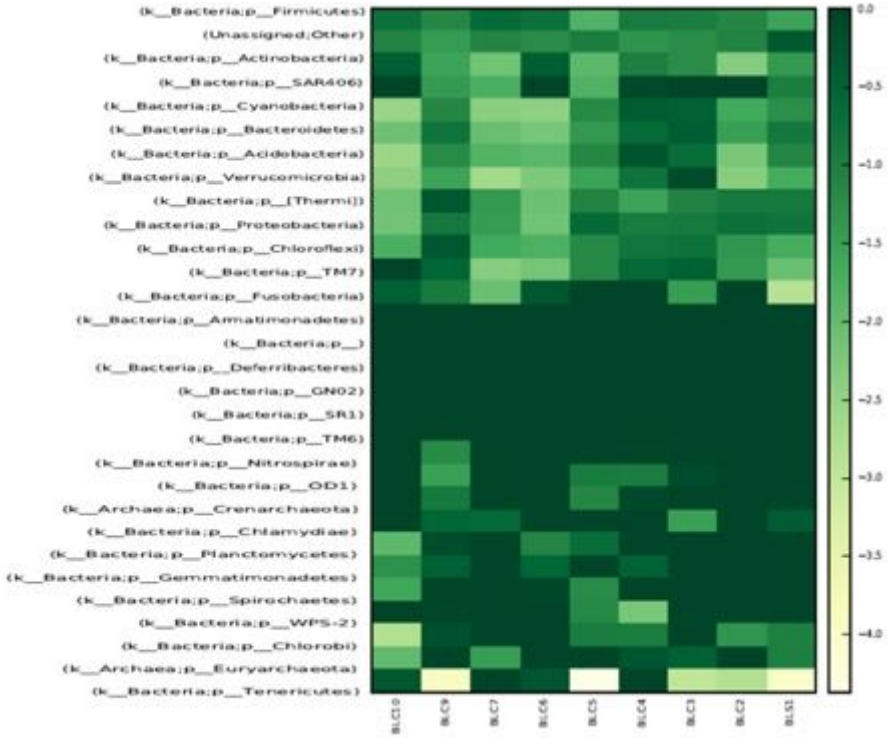

Figure 2

The heat graph showed the number of phyla in each sample in terms of color intensity. Collectively, the maximum bacterial diversity was detected in BLC3 and BLC4 while least was observed in BLS1 as it represented lesser color intensity areas.

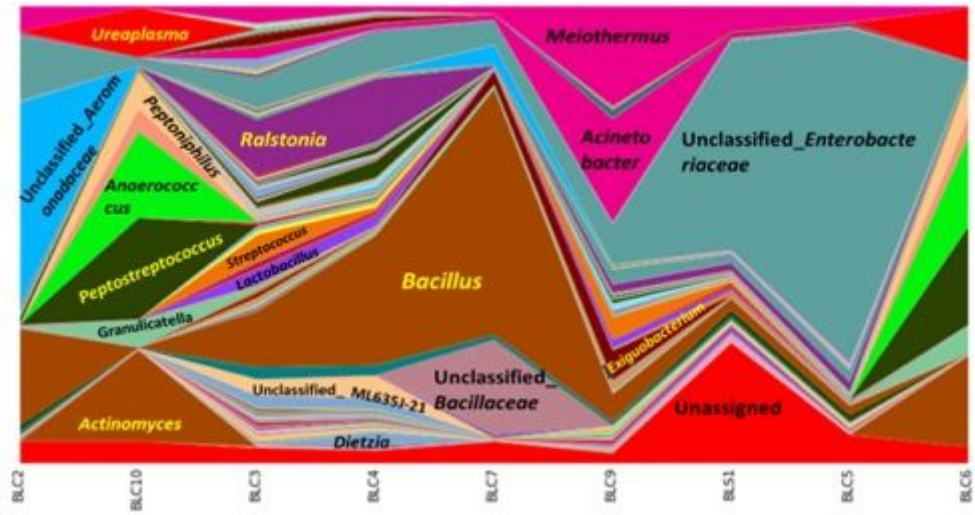

Figure 3

The bacterial genera observed in human bladder biopsy samples as a result of 16S rRNA gene analysis through QIIME. The result has shown in the form of an area density graph: prominent genera observed in samples were labelled in figure.

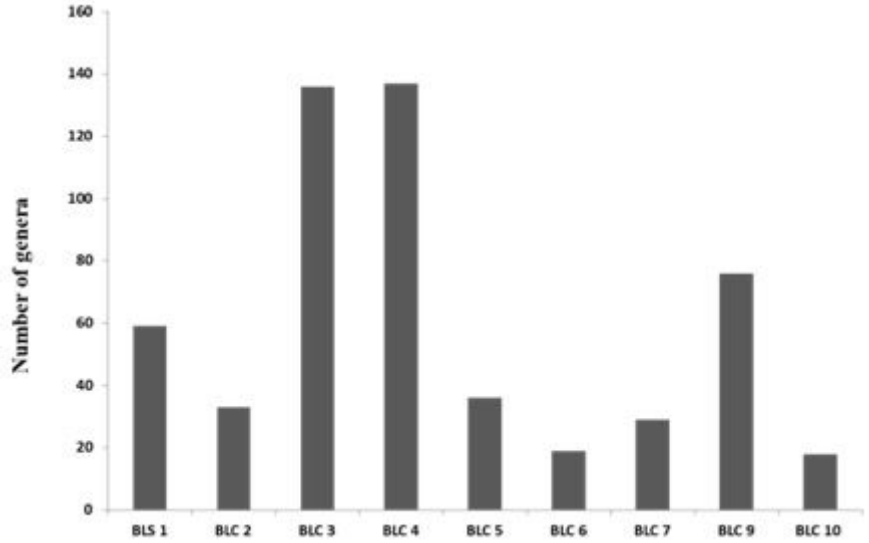

Figure 4

The number of genera observed in each sample.

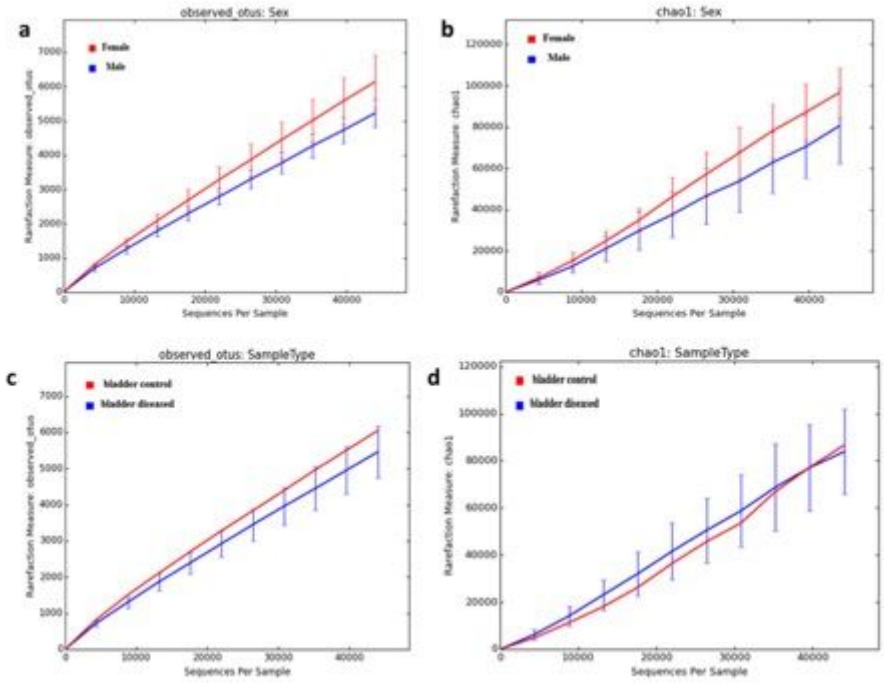

Figure 5

Rarefaction curves of 16S rRNA gene sequences formed from biopsy samples: sex (males and females) and sample types (Control and diseased samples). Line represents the average value for each subject and standard deviation has shown in terms of error bars. a Observed OTU based on sex b Rarefaction curves (Chao 1) based on sex c Rarefaction measure based on Sample type d Rarefaction curves (Chao 1) based on Sample type.

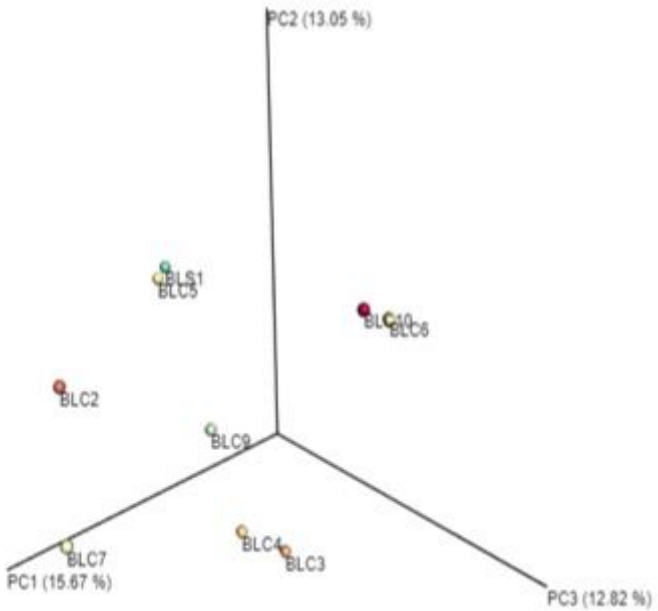

Figure 6

Principal coordinate analysis (PCoA) plot of the unweighted Unifrac matrix based on Sample ID: P value is 0.8889 showed insignificant differences between samples.