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PROFILING OF BACTERIAL SPECIES FROM COVID-19 FAECAL SAMPLES IN KURDISTAN REGION-IRAQ

Mohammed A. Hama-Ali [a], Ayad H. Hasan [a, b, *]

[a] Dept. of Medical Microbiology, Faculty of Science and Health, Koya University, Koya KOY45, Kurdistan Region -F.R. Iraq.
[b] Dept. of Biomedical Sciences, College of Health Technology, Cihan University-Erbil, Kurdistan Region, Iraq.
(ayad.hasan@koyauniversity.org)

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ABSTRACT:

The invasion of intestinal cells by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may have an impact on the gut bacteria. This study investigated the alteration of gut bacteria during SARS-CoV-2 viral infection and after recovery. Faecal samples were collected from ten RT-PCR-confirmed COVID-19 patients and five healthy participants (served as a control group) from November 21st, 2021, to April 1st, 2022. The faeces samples were collected three times, at the time of infection, after seven days of the infection, and on day fifty after clearance of SARS-CoV-2. Serum samples were used to perform serological tests for the control group and COVID-19 survived patients. Pure culture techniques, classical, and molecular approaches were used to isolate and identify the bacterial population in the collected faeces. The faecal bacterial communities of patients with COVID-19, those who recovered, and the five healthy people were compared. Significant alteration in culturable gut bacteria was observed in COVID-19 patients compared to the control group. This alteration was expressed by the existence of four bacterial species, which were *Escherichia fergusonii*, *Citrobacter portucalensis*, *Comamonas kerstersii*, and *Shigella flexneri*. In addition, two respiratory tract-associated bacterial pathogens, *Klebsiella pneumoniae* and *Klebsiella aerogenes* were recovered from the faecal samples of 40% of COVID-19 patients. The results even revealed that *Staphylococcus aureus* was more prevalent in faeces samples from those with SARS-CoV-2 infections than the healthy individuals. Faecal analysis of COVID-19 patients showed the existence and elevation of pathogenic bacteria in the large intestine in comparison to the healthy group. Further studies are required to highlight how an alteration of gut microbiomes affects the course of COVID-19 infection.

KEYWORDS: Gut bacteria, COVID-19, 16S RNA gene, SARS-CoV-2, Comamonas kerstersii.

1. INTRODUCTION

A consistent relationship (symbiosis) between the human body and its natural microbiota starts at delivery. The sustainability of overall health and well-being depends heavily on this relationship, which can be communalistic, mutualistic, or pathogenic (Ogunrinola *et al.,* 2020). The species that make up the microbiota have developed considerably, and they actively respond to their habitats, such as the skin, the mucosa, the gastrointestinal tract, the respiratory tract, the urogenital tract, and the mammary gland within the human body (Whiteside *et al.,* 2015). The human gut alone contains more than 100 trillion bacteria that are affected by various factors, including delivery method, baby feeding practices, lifestyle, medications, food, age, and the host's genetic makeup (Wang *et al*., 2017). These microbes play significant roles in metabolism, immunity development, and defence against pathogens, all of which have a direct or indirect impact on many human physiological processes (Robinson *et al.,* 2010; Covasa *et al.,* 2019). The training of the host's immunity system, food digestion, control of gut; endocrine and neurological signals, modification of medication action and metabolism, elimination of toxins and production of various chemicals (Fan and Pedersen 2020) such as bile acids, lipids, amino acids, vitamins, and short-chain fatty acids that have an impact on the host are all key functions of the gut microbiome (Brestoff and Artis 2013; Kho *et al.,* 2018). Changes in the composition and function of intestinal microorganisms, known as dysbiosis, are linked to several diseases, including neurologic, respiratory, metabolic, hepatic, and cardiovascular conditions, as well as more localized gastroenterological ailments (Fan and Pedersen 2020). It is well documented that the gut and respiratory tract have been connected to modulating immune responses at the time of disease development in the respiratory tract, which in some cases progresses to secondary bacterial infections (Fanos *et al.,* 2020; Hanada *et al.,* 2018; Yildiz *et al.,* 2018).

It has been shown that 20% of the respiratory syndrome coronavirus 2 cases had gastrointestinal (GI) symptoms, such as diarrhoea, vomiting or abdominal pain (Huang *et al.,* 2019; Chen *et al.,* 2020; Liang *et al.,* 2020; Cheung *et al.,* 2020) and the presence of SARS-CoV-2 virus in the stools and anal specimens of nearly 50% of COVID-19 cases suggests that the gastrointestinal tract could be an extra-pulmonary site for viral activity and replication (Wölfel *et al.,* 2020).

Recent studies on Chinese COVID-19 patients have found a state of dysbiotic microbiota, decreasing the number of favourable commensals, particularly those that generate short-chain fatty acids (SCFAs), such as those from the *Lachnospiraceae* and *Ruminococcaceae* families, and increasing the number of opportunistic pathogens or pathobionts (Gu *et al.,* 2020; Zuo *et al.,* 2020; Chen *et al.,* 2021; Zuo *et al.,* 2021).

Understanding the host microbial perturbations that SARS-CoV-2 causes is urgently needed might be due to its ability to alter the body's response to infection and the effectiveness of different immunological therapies like vaccinations. Consequently, in this

* Corresponding author

study, we aimed to examine how the gut flora of COVID-19 changed over time during the infection and after clearance, using classical and molecular methods.

2. MATERIAL AND METHODS

2.1 Sample collection

Faecal samples were collected from ten COVID-19 patients who were positively confirmed by a local hospital (Central Laboratory of Koya and Shahid Dr Hemn Teaching Hospital) using RT-qPCR. Stool samples were also collected from five healthy persons as a control group, which did not receive any antibiotics during the time of the study (three months). From the COVID-19 patients, faecal samples were collected at three different time points: at the time of infection; after 7 days following the infection; and 50 days after the patient had recovered. The samples were processed within 4 hours in the laboratory for culturing. Individuals of the control group and patients after clearance were subjected to a serological test to measure any trace of the previous infection with SARS-CoV-2 by calculating IgG and IgM levels using a mini-VIDAS device at the REGA specialist laboratory in Sulaymaniyah. Then the stool samples were collected from the control group individuals, who are relatives of the patients once without defining the time frame.

2.2 Classical identification

2.2.1 Isolation: To prepare a pure culture from the samples, a loopful of stool was homogenized in 1ml of sterilized nutrient broth by vertex, and then 50 µl was spread on: Nutrient agar, MacConkey agar, Eosin methylene blue agar, and Mannitol salt agar. All the inoculated plates were incubated overnight at 37°C (Murray *et al.,* 2006). Single different colonies with different characteristics and morphology in each plate set were subjected to a pure culture technique and then stored at -80°C in 25% glycerol for further investigations.

2.2.2 Staining and biochemical tests: The isolated colonies were stained with standard Gram stain and examined under a compound microscope at 100X. The following biochemical tests were performed for the isolate's phenotypic identification: indole, methyl red, Voges Proskauer and citrate utilization (IMVC), triple sugar iron agar (TSI agar), urease, oxidase, catalase, and motility (Atlas *et al*., 1995, Smith and Hussey 2005 and Cappuccino and Welsh, 2019).

2.3. Molecular identification

2.3.1 Broth culture preparation: To extract genomic DNA from a representative of each bacterial group, a loopful of certain bacteria was inoculated in a 15 ml falcon tube containing 5 ml of nutrient broth, incubated with shaking at 150 rpm for 24 hrs at 37oC.

2.3.2 Genomic DNA extraction: DNA was extracted from nineteen different bacterial samples that were representing their either genus group or stand-alone genus using FavorPrep genomic DNA mini kit (Favorgen) applying the guidelines provided by the manufacturer. Nanodrop (Thermo Scientific NanoDrop 2000. SN. 6113) was used to check the quantity and purity of the extracted DNA, which was subsequently kept at -20°C. The genomic DNA was subjected to further analysis by running 60 ng on 1 % agarose gel for 60 min at 80 V.

2.3.3 Amplification of 16S rDNA by standard PCR: A ~1515 bp of 16S rDNA was amplified using a PCR approach with a final volume of 30 µl reaction including 15 µl of 2X Add Taq Master (Addbio), 5 pmol (1 µl) of each forward (P1F-TGAAGAGTTTGATCATGGCTCAG) and reverse (P1R-TTCCCCTACGGTTACCTTGT) primers, and 20ng (1 µl) genomic DNA. The volume was completed by adding 12µl of nuclease-free water (**Figure 1**).

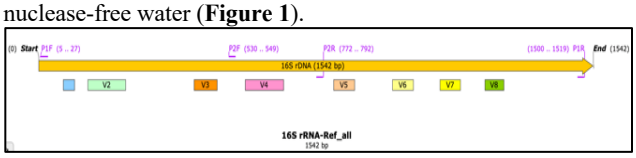

Figure 1: Primer's binding sites on 16S rDNA. P1F and P1R primes provide a ~1515bp PCR amplicon, while P2F and P2R provide a ~265bp amplicon including the V4 region. The figure was generated using data from (Chakravorty *et al.* 2007)

The PCR was carried out using a BIO-RAD and Corbett thermal cycler and was configured as follows for M1, M2, M4, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15, M16, M17, and M18 samples: Initial denaturation at 95°C for 5 minutes, then 27 cycles of 30 seconds at 95°C for denaturing, 25 seconds for annealing at 58°C, 60 seconds for the extension at 72°C, and 5 minutes for the final extension at 72°C.

However, samples M3, M5, and M19 were amplified with different cycling conditions, which were 33 cycles at 95°C for 40 seconds as the denaturation step, 59.1°C for 40 seconds as the annealing step and 60 seconds at 72°C as the final extension with same initiation denaturation and final extension conditions as mentioned above.

2.3.4 16S rDNA amplicon integrity: To investigate the existence of the V4 region, a total of 30µl PCR reactions was set up containing 1 µl of the PCR amplicons of the 16S rDNA (Section 2.3.3), 1 µl (5 pmol) P2F primer (GTAATACGGAGGGTGCAAGC), 1 µl (5 pmol) P2R primer (TCTAATCCTGTTTGCTCCCCA), 15 µl of 2X Add Taq Master (Addbio), then the volume was completed by adding 12 µl of nuclease-free water (Figure 1). The resulting PCR amplicon must be ~ 263 bp. The PCR was carried out using the BIO-RAD and Corbett thermal cycler and was conducted as follows: Initial denaturation at 95°C for 5 minutes, then 30 cycles of 35 seconds at 95°C for denaturing, 30 seconds for annealing at 58°C, 50 seconds for the extension at 72°C, and 5 minutes for the final extension at 72°C.

2.3.5 Agarose gel electrophoresis: To check the availability of the right PCR amplicons, 2 µl of PCR amplicons were electrophoresed on a 1% agarose gel with 0.07% EtBr along with a 100 bp DNA ladder (Genedrix) and run in 1X TBE buffer at 80 V for 60 minutes to validate that the targeted gene was amplified correctly. Following the run, visualization and photographing of the DNA molecules were done using UV Gel Imager SynGene 1409.

2.3.6 Partial 16S rDNA sequencing: The resulting ~ 1515 bp PCR amplicon was sent out to Macrogen Inc, a South Korean company for sequencing using P2R to include the V4 region.

2.3.7 Quality of the sequenced products: The DNA baser assembler program was used to perform sequence quality, analysis, and editing. The start and end of the sequence were trimmed to assess the quality of the sequence.

2.4 Bacterial identification

To classify the bacterial isolates independently, the EzBioCloud was used to compare the 16S rDNA sequence to previously discovered bacterial DNA sequences (Yoon *et al*., 2017).

3. RESULTS

Ten verified COVID-19 patients aged (26-52) and five healthy controls aged (25-45) were recruited to be followed up on their most common bacterial alteration. All patients were diagnosed with moderate COVID-19 accompanied by cough and shortness of breath, although only one had a gastrointestinal (GI) symptom of diarrhoea (Patient number 5). None of the patients was suffered from chronic diseases, vaccinated against SARS-CoV-2, and experienced GI problems (Table S1). Triple stool samples were taken from each patient at three different timelines. The first, second and third stool collections were named baseline, illness period and endline, respectively. The patient's recovery from COVID-19 and the healthy control's history of COVID-19 infection was confirmed and investigated by RT-qPCR and serologically. All the COVID-19 surviving individuals displayed a negative attitude toward the IgM test, which is a sign of full recovery from the infection and a positive attitude against IgG, which is an indication of past infection. On the contrary, the healthy controls serological tests were negative against IgM and IgG (Table S2).

3.1 Standard identification of the bacterial isolates

Eighty-nine different bacterial isolates were isolated from the stool samples including the controls. They were grouped into 6 groups as follows: *E. coli* (47 isolates from the patients and 8 isolates from the controls), *Klebsiella spp.* (8 isolates from the patients and 0 isolates from the controls), *Shigella spp.* (3 isolates from the patients and 0 isolates from controls), *Citrobacter* spp (only 2 isolates from patients and 0 from controls), *Enterobacter* spp. (1 isolate from patients and 1 isolate from controls), and *Staphylococcus aureus* (14 isolates from patients and 2 isolates from controls). However, we could not identify 3 bacterial isolates based on their cultural characteristics and biochemical tests (Tables S3 and S4).

3.2 Bacterial Identification at the molecular level

To assess the extracted DNA from the bacterial samples, gel electrophoresis was employed and Nanodrop was used. No degraded trace was observed in all the genomic DNA samples (Figure 2) which were supported by the Nano-drop results with average purities (A_{260}/A_{280}) of 1.87 (Table S5).

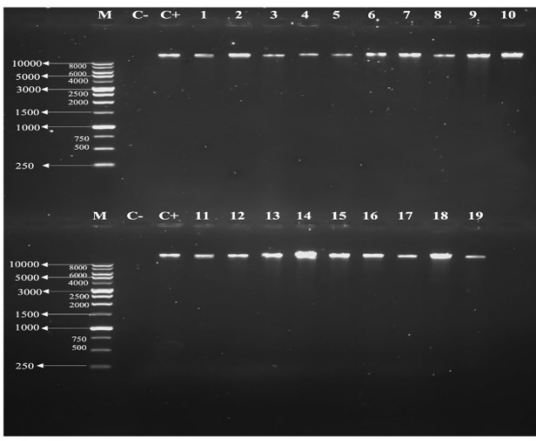

Figure 2: Agarose gel electrophoresis analysis of genomic DNA extracted from the bacterial genera.

The DNA fragments were investigated with 1 % agarose gel. M; 1Kb DNA marker from Genedirex, C^-; negative controlcontrol, which is distilled water, C^+; positive control in which the genomic DNA extracted from *E. coli* strain ATCC 25218; lanes (1-19) represent the bacterial sample number in which the genomic DNA was extracted from. The wells contain high molecular weight and genomic DNA with yields of on average 85 µg and average purities of 1.87 at (A_{260}/A_{280}). No degradation was observed in all samples. The negative control result verified that the DNA was pure.

The predicted size of the DNA fragment (~1515 bp) was effectively amplified from bacterial isolate template DNA, and no PCR products were observed in the negative controls (Figure 3). To ensure the amplified PCR products contain the V4 region, P2F and P2R primers were used to target downstream and upstream of the V4 region, respectively. The predicted PCR products of ~ 263 bp were generated for all the samples (Figure 4). To determine the species of each bacterial isolate, the 16S rRNA gene PCR amplicons that have been produced from each isolate using P1F and P1R primers were sequenced using the reverse primer P2R.

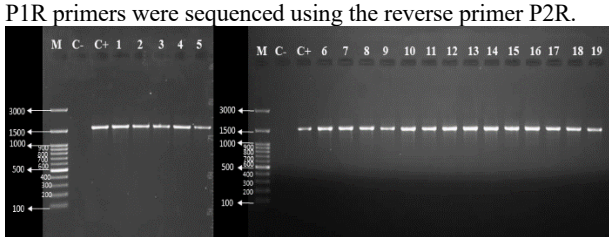

Figure 3 Partial amplification of 16S rDNA using P1F and P1R primers. Lanes M, C^- and C^+ represent a 100bp DNA marker (Genedirex), a negative control that has been run without a DNA template and a positive control that has been run using DNA from E. coli strain ATCC 25218, respectively. Lanes 1 through 19 shows ~1515bp of PCR amplicons generated using a DNA template from M1 through M19, respectively.

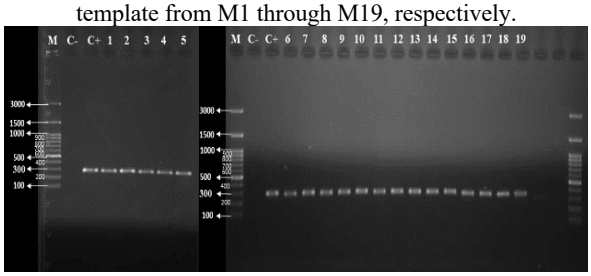

Figure 4 16S rDNA verification using P2F and P2R primers. Lanes M, C^- and C^+ represent a 100bp DNA marker (Genedirex), a negative control that has been run without a DNA template and a positive control that has been run using DNA from E. coli strain ATCC 25218, respectively. Lanes 1 through 19 shows ~263bp of PCR amplicons generated using a PCR template (from section 2.3.3) from M1 through M19, respectively.

3.3 Sequencing and DNA quality

Depending on whether the DNA sequencing was reliable, the 19 investigated samples were considered for further investigation on their high-quality values (QV), which were above 40.

3.4 Bacterial genera

To specify the taxonomic origin of the nineteen 16S rDNA sequences, independent computerized alignments were performed versus accessible prokaryotic sequences of 16S rDNA employing EzBioCloud (Yoon *et al*., 2017). All nineteen individual sequences were found to be identical to previously cultivated bacteria (Table 1).

Table 1 Identification of selected pure bacterial colonies using classical and molecular approaches.

Sample No	Expectation	Molecular identification	Accession Number
M1	*Shigella* spp	*Shigella dysenteriae*	OP808014
M2	*Citrobacter* spp	*Citrobacter portucalensis*	OP808015
M3	??	*Comamonas kerstersii*	OP808016
M4	*Shigella* spp.	*Shigella dysenteriae*	OP808017
M5	??	*Comamonas kerstersii*	OP808018
M6	??	*Shigella flexneri*	OP808019
M7	*Enterobacter*	*Enterobacter cloacae*	OP808020
M8	*Shigella* spp.	*Shigella dysenteriae*	OP808021
M9	*E. coli*	*Escherichia fergusonii*	OP808022
M10	*Klebsiella* spp.	*Klebsiella aerogenes*	OP808023
M11	*Klebsiella* spp	*Klebsiella pneumoniae*	OP808024
M12	*E. coli*	*Escherichia fergusonii*	OP808025
M13	*E. coli*	*Escherichia fergusonii*	OP808026
M14	*Enterobacter*	*Enterobacter cloacae*	OP808027
M15	*Shigella* spp.	*Shigella sonnei*	OP808028
M16	*E. coli*	*Escherichia fergusonii*	OP808029
M17	*E. coli*	*E. coli*	OP808030
M18	*E. coli*	*Escherichia fergusonii*	OP808031
M19	*S. aureus*	*S. aureus*	OP808032

Table 1 shows the results of traditional identifications against the molecular method. Even though certain species could not be identified by the classical identification chosen for this study, some classical identifications were successfully comparable to molecular identifications.

Following biochemical tests and molecular confirmation, eleven distinct bacterial species that belong to seven different genera of bacteria were identified; the percentage of bacterium species out of 89 were as follows; 47% *Escherichia coli* (42 isolates), 18% *Staphylococcus aureus* (16 isolates), 12% *Escherichia fergusonii* (11 isolates), 6% *Klebsiella pneumoniae* (5 isolates), 4% *Shigella dysenteriae* (4 isolates), 3% *Klebsiella aerogenes* (3 isolates), 2% *Enterobacter cloacae* (2 isolates), 2% *Citrobacter portucalensis* (2 isolates), 2% *Comamonas kerstersii* (2 isolates), 1% *Shigella flexneri* (1 isolate), and 1% *Shigella Sonni* (1 isolate) (Figure 6).

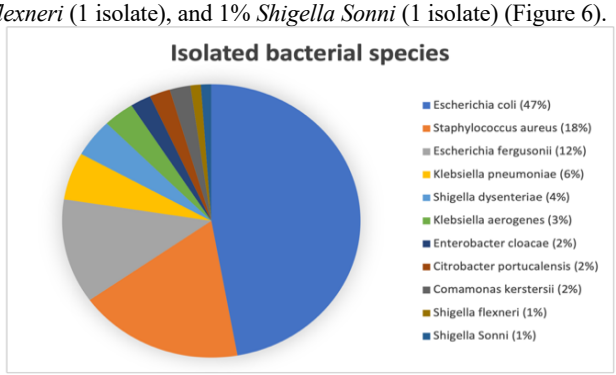

Figure 6 Bacterial species that have been isolated from the patients.

3.5 The faecal bacterial profile associated with COVID-19 infection

To acquire more about the change in the gut bacterial profile in COVID-19 patients, we compared the gut bacteria of faecal samples with a characteristic of COVID-19 infection to those from the control group. The gut bacterial composition of faeces samples from the COVID-19 patients and the five healthy controls was analyzed after faecal culture, conventional identification, and 16S rDNA sequencing and identification. We found that the patients' faeces included the following bacterial species that weren't present in the control group: *Escherichia fergusonii*, *Citrobacter portucalensis*, *Comamonas kerstersii*, *Shigella flexneri*, *Klebsiella pneumoniae*, and *Klebsiella aerogenes*. Among these species, *Klebsiella pneumoniae* and *Klebsiella aerogenes* have been linked to bacterial infections that affect the respiratory tract. Compared to the control group, *Staphylococcus aureus* colonized all of the patients' faeces in large numbers. Thus, these results indicate that COVID-19 affects the bacterial population in the gut.

4. DISCUSSION

The classical and molecular identification were in agreement in determining most bacterial genera, except that the latter identified all the samples down to the species level (Rhoads *et al.,* 2012). In addition, the conventional approach could not identify samples M3, M5, and M6 based on the biochemical tests. The above results suggest that the molecular approach for bacterial identification has an advantage over the biochemical methods. In this work, we sought to determine if alteration of gut bacterial population was linked to infection with COVID-19 throughout the infection and curing period in comparison with some control and after curing. *S. aureus* was shown to be significantly linked with SARS-CoV-2 faecal samples in most of the patients after seven days of the infection. However, it has been lost in 75% of the patients after recovery from the viral infection. Six bacterial species including *Escherichia fergusonii*, *Klebsiella pneumoniae*, *Klebsiella aerogenes*, *Citrobacter portucalensis*, *Comamonas kerstersii*, and *Shigella flexneri* revealed a considerable increase in the COVID-19 survived patients compared to the healthy group. The presence of *Klebsiella* spp. in the COVID-19 gut supports the transit or transfer of extra-intestinal microorganisms into the gut. The above result is supported by (Yildiz *et al.,* 2018) and (Groves *et al.,* 2018) studies, which demonstrated that the population of the gut microbiota may be altered by pulmonary viral infections like influenza and respiratory syncytial virus. Considering the baseline abundance of opportunistic pathogens, gut microbiota may have a significant impact on how severe COVID-19 is and the existence of the virus in the gut of the host. Lack of determination of beneficial bacterial species in COVID-19 survived individuals even after clearance, indicating that SARS-CoV-2 viral infection may be linked with a greater long-term harmful impact on the gut microbiome (Liu *et al.,* 2022). All these results point to the possibility that the composition of a patient's most common bacteria may have an impact on how they react to and are susceptible to SARS-CoV-2 infection. The small sample size of this pilot study is a significant drawback. Although larger validation studies are necessary before establishing a correlation link between COVID-19 and gut microbiota, this pilot study provides the first information on the impact of SARS-CoV2 infection on the composition and dynamics of the most common gut bacteria in the Kurdistan region. So, to clarify the significance of microbiome alterations in SARS-CoV-2 infection and survived individuals, additional studies should be conducted with larger size of samples and analysing gut bacterial 16S rRNA genes using a metagenomic approach.

5. CONCLUSION

Our data indicate that COVID-19 patients would experience an alteration in their gut microbiome, which may in turn play a significant effect on how severe the infection is. Therefore, concentrating on the best technique to restore the gut microbiome balance to its healthy state may help COVID-19 patients recover more quickly and effectively. The Faculty of Science and Health's research ethics committee at Koya University approved the research proposal on December 20, 2021.

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Supplementary Materials

Table S1 Summary of the completed questionnaire forms.

Variables		**COVID-19 cases**		
Name:		**1st**	**2nd**	**3rd**
Gender	F: 4 M: 6			
Age	26-52			
Do you suffer from a chronic illness diagnosed by a physician?	No/ 10			
Hypertension	No/ 10			
Diabetes	No/ 10			
Heart disease	No/ 10			
Obesity	No/ 10			
When did the symptoms appear?	No/ 10			
Others	No/ 10			
Are you taking any medication?	No/ 10			
Have you used antibiotics in the last three months? If yes, name it.	No/8 Not sure/ 2			
Please self-rate your current health status		not bad/ 4 bad/ 2 good/ 4	not bad/ 5 bad/4 good/ 1	good/ 10
Vaccination status	No/ 10			
When did you know that you are suffering from COVID-19 after the appearance of the symptoms?	B1D/ 7 B2D/ 2 B3D/ 1			
Were you tested for COVID-19 in the past 14 days?	B2D/ 7 B3D/3			
Have you travelled outside of your residential country/ area?	No/10			
Have you directly or indirectly contacted patients suffering from COVID-19?	No/5 Yes/ 5			
Have family cluster outbreak	No/7 Yes/ 3			
When you have been diagnosed with COVID-19	Sulaymanyah/ 6 Koya/ 4			
Symptoms at admission				
Fever (temperature on administration)		Yes/ 10 No/0	Yes/4 Sometime/ 6 No/ 0	Yes/ 0 No/10
Gastrointestinal symptoms				
Type of the Stool sample				
Soft		3	8	8
Semi soft		3	1	0
Hard		2	1	2
Liquid		2	0	0
Respiratory symptoms				
Cough		Yes/ 10 No/ 0	Yes/ 10 No/ 0	Yes/ 2 No/8
Sputum		Yes/ 3 No/7	Yes/ 10 No/ 0	Yes/ 0 No/10
Rhinorrhoea		Yes/6 No/ 4	Yes/3 No/7	Yes/ 0 No/10
Shortness of breath		Yes/ 8 No/ 2	Yes/ 9 No/1	Yes/ 0 No/10
Antibiotic therapy at presentation,				
Amoxicillin Clavulanate			6	

Cephalosporin				
Azithromycin			6	
Tetracycline				
Levofloxacin			1	
Ceftriaxone				
Moxifloxacin			2	
Meropenem				
Other				
Antiviral therapy,			1	
Lopinavir-Ritonavir				
Ribavirin				
Interferon beta-1b				
Others				
Analgesics				
Paracetamol			6	
Ibuprofen				

B*= Before
D*= Days

Table S2 Serological result of IgG and IgM antibodies of COVID-19 patients and control group.

Sample No.	IgM IU/ml	IgG IU/ml	Normal range IU/ml	Result
1	Negative (0.94)	Positive (15.62)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
2	Negative (0.87)	Positive (8.97)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
3	Negative (0.80)	Positive (7.10)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
4	Negative (0.93)	Positive (21.4)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
5	Negative (0.84)	Positive (13.34)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
6	Negative (0.87)	Positive (10.87)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
7	Negative (0.89)	Positive (6.89)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
8	Negative (0.91)	Positive (9.25)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
9	Negative (0.89)	Positive (17.42)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
10	Negative (0.93)	Positive (10.2)	Positive: ≥ 1.0 Negative: < 1.0	Previous infection (Recovered)
C1	Negative (0.83)	Negative (0.85)	Positive: ≥ 1.0 Negative: < 1.0	COVID-19 free individual
C2	Negative (0.91)	Negative (0.89)	Positive: ≥ 1.0 Negative: < 1.0	COVID-19 free individual
C3	Negative (0.65)	Negative (0.72)	Positive: ≥ 1.0 Negative: < 1.0	COVID-19 free individual
C4	Negative (0.74)	Negative (0.81)	Positive: ≥ 1.0 Negative: < 1.0	COVID-19 free individual
C5	Negative (0.79)	Negative (0.85)	Positive: ≥ 1.0 Negative: < 1.0	COVID-19 free individual

Table S3 List of biochemical test results and bacterial Spp. isolated from COVID-19 Patients.

No.	Time point	Colonies	Biochemical tests									Classical ID	Molecular ID	Last Diction
			Oxidase	Catalase	TSI	IMVIC				Urea	Motility			
						Indole	Methyl red	Voges-proskauer	Simmon,s citrate					
1	A	1	-	+	A/A	-	-	+	+	-	-	*Klebsiella* spp.		*Klebsiella aerogenes*
		2	-	+	A/A	+	+	-	+	+	+	May be *E. coli*	*Escherichia fergusonii*	*Escherichia fergusonii*
	B	1	-	+	A/A	-	+	-	+	-	-	*Klebsiella* spp.		*Klebsiella aerogenes*
		2	-	+	A/A	+	+	-	-	-	+	Late lactose fermenter *E. coli*		*Escherichia fergusonii*
		3										*S. aureus*		*S. aureus*
	C	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3	-	+	A/A/G	+	+	-	-	-	+	Late lactose fermenter *E. coli*	*Escherichia fergusonii*	*Escherichia fergusonii*
		4										*S. aureus*		*S. aureus*
2	A	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
	B	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	May be *E. coli*		*E. coli*
	C	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
3	A	1	-	+	A/A/G	-	-	+	+	Weak+	-	*Klebsiella* spp.		*Klebsiella pneumoniae*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	B	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A	-	-	+	+	-	-	*Klebsiella* spp	*Klebsiella aerogenes*	*Klebsiella aerogenes*
		3										*S. aureus*		*S. aureus*
	C	1	-	+	A/A/G	+	+	-	-	-	+	Mucoid *E. coli*		*Escherichia fergusonii*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
4	A	1	-	+	A/A/H2S	-	+	-	+	-	+	May be *Citrobacter* spp.		*Citrobacter portucalensis*
		2	-	+	A/A/G	+	+	-	-	-		*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
	B	No growth												
	C	1			A/A/H2S	-	+	-	+	+		*Citrobacter* spp. or *Proteus* spp.	*Shigella* dysenteriae	*Shigella* dysenteriae
		2	-	+	A/A/H2S/G	-	+	-	+	-	+	May be *Citrobacter* spp.	*Citrobacter portucalensis*	*Citrobacter portucalensis*
		3	– -	+	A/A/G	-	+	+	+	Weak +	-	*Klebsiella* spp.		*Klebsiella pneumoniae*

		4	-	+	A/A/G	-	+	+	+	Weak +	-	*Klebsiella* spp.		*Klebsiella pneumoniae*
5	A	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	-	+	+	+	Weak +	-	*Klebsiella* spp.		*Klebsiella pneumoniae*
	B	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*	*E. coli*	*E. coli*
		3										*S. aureus*		*S. aureus*
	C	1	-	+	A/A/G	-	-	+	+	+	-	*Klebsiella* spp.	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
		2	-	+	A/A/G	+	+	-	-	-	+	Late lactose fermenter *E. coli*		*Escherichia fergusonii*
6	A	1	-	+	A/A	+	+	-	-	-	+	Late lactose fermenter *E. coli*		*Escherichia fergusonii*
		2	-	+	A/A/G	+	+	-	-	-		*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
	B	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A	+	+	-	-	-	+	May be Mucoid *E. coli*		*Escherichia fergusonii*
	C	1	-	+	A/A/G	+	+	-	-	-	+	Late lactose fermenter *E. coli*	*Escherichia fergusonii*	*Escherichia fergusonii*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
7	A	1	+	+	K/K	-	+	-	-	-	+	??????	*Comamonas kerstersii*	*Comamonas kerstersii*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3	-	+	K/A	+	+	-	-	-	-	May be *Shigella* spp.		*Shigella* dysenteriae
		4										*S. aureus*		*S. aureus*
	B	1	-	+	A/A	+	+	-	-	-	+	May be Mucoid *E. coli*	*Escherichia fergusonii*	*Escherichia fergusonii*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	C	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
8	A	1	-	+	K/A	+	+	-	-	-	-	Inactive *E. coli* or *Shigella* spp.	*Shigella* dysenteriae	*Shigella* dysenteriae
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3										*S. aureus*		*S. aureus*
	B	1	-	+	A/A	+	+	-	-	-	+	Late lactose fermenter *E. coli*	*Escherichia fergusonii*	*Escherichia fergusonii*
		2	+	+	K/K	-	+	-	-	-	+	?????	*Comamonas kerstersii*	*Comamonas kerstersii*
		3	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	C	1	-	+	A/A/G	+	+	-	-	-	+	May be *E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
9	A	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	B	1	-	+	A/A	+	+	-	-	-	+	Late lactose fermenter *E. coli*		*Escherichia fergusonii*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*

		3	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		4										*S. aureus*	*S. aureus*	*S. aureus*
	C	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		3	-	+	K/A/G	+	+	-	-	-	-	?????	*Shigella flexneri*	*Shigella flexneri*
10	A	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	-	-	+	+	-	+	Inactive *E. coli* or *Klbsiella* spp.	*Enterobacter cloacae*	*Enterobacter cloacae*
		3										*S. aureus*		*S. aureus*
	B	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
		2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	C	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*

Table S4 List of biochemical test results and bacterial Spp. isolated from control group

No.	**Colonies**	**Biochemical tests**									**Classical ID**	**Molecular ID**	**Last decision**
		Oxidase	Catalase	TSI	IMVIC				Urea	Motility			
					Indole	Methyl	Voges-	Simmon,s					
1	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		
	2										*S. aureus*		
2	1	-	+	K/A	-	+	-	-	-	-	Inactive *E. coli* or *Shigella* spp.	*Shigella sonnei*	*E. coli*
		-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*S. aureus*
3	1	-	+	K/A	+	+	-	_ -	-	-	Inactive *E. coli* or *Shigella* spp.	*Shigella dysenteriae*	*Shigella sonnei*
	2	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	3										*S. aureus*		*Shigella dysenteriae*
4	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*
	2	-	+	A/A	+	+	-	-	-	+	*E. coli*		*S. aureus*
5	1	-	+	A/A/G	+	+	-	-	-	+	*E. coli*		*E. coli*

Table S5 Quantity and quality of bacterial extracted DNA.

No	Code	Sample No	Concentration ng/µl	260/280 ratio
1	M1	4C-1	30	1.8
2	M2	4C-2	29	1.89
3	M3	7A-1	78	2.0
4	M4	8A-1	25	1.89
5	M5	8B-s	36.5	1.97
6	M6	9C-3	20	1.85
7	M7	C5-3	35	1.82
8	M8	C3-1	31	1.9
9	M9	7B-2	20	1.78
10	M10	3B-2	23	1.81
11	M11	5C-1	25	1.84
12	M12	8B-2	30	1.88
13	M13	1C-3	28	1.90
14	M14	10A-2	34	1.89
15	M15	C2-1	31	1.78
16	M16	1A-2	25	1.89
17	M17	5C-2	29	1.87
18	M18	6C	38	1.91
19	M19	9B	19	1.93