

Two Year Cross Sectional Study on Chronic Brucellosis in Central Iran

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Abstract: Brucellosis is a zoonotic infectious disease with possibility of chronic symptoms. The purpose of this study was to investigate the prevalence of chronic brucellosis and assess the epidemiologic, laboratory and clinical features of patients with chronic brucellosis in Central Iran. This cross-sectional study was conducted on all patients with chronic brucellosis referred to Valiasr Hospital of Arak (July 2011-July 2013). A demographic, epidemiological and clinical questionnaire was filled out for all patients; they underwent bone marrow aspiration and serologic investigations. Blood and bone marrow were subjected to microbiological culture, serology was performed and bone marrow was analyzed by PCR. Results: Among 1047 patients with brucellosis, 15 patients (1.5%) were diagnosed with chronic brucellosis clinically and based on serologic tests. Of them, 1 (6%), 1 (6%) and 5 (33%) patients had a positive blood culture, bone marrow culture or bone marrow PCR, respectively. Living in a village, contact with meat, continuous consumption of local unpasteurised milk and rifampin + doxycycline antibiotic usage were associated with chronic Brucellosis. Conclusions: Our study showed that 33.3% of chronic brucellosis patients were positive in bone marrow PCR assay. As a result, the examination and follow-up of patients with chronic brucellosis using microbiological additional tests such as PCR is recommended.

Key words: Chronic Brucellosis • Clinical Features • Laboratory Aspects

INTRODUCTION

Brucellosis is one of the most important zoonotic diseases that continue to impact economy and human health. It affects humans in many developing countries including the Middle East and Latin America where it is still endemic [1]. The World Health Organization reports an annual incidence of human brucellosis of less than one to 78 cases per 100,000 population in the Middle East, with six countries reporting an annual total of more than 90,000 cases [2]. Brucellosis remains an important public health problem in Iran, causing serious complications and significant morbidity. The commonest etiological agent in our region is *Brucella melitensis* [3, 4]. The transmission of *Brucella spp* from infected animals to humans occurs either by occupational contact or the consumption of

contaminated animal products, especially milk, cream, butter and fresh cheese [5, 6]. The disease is characterized by fever, arthralgia, sweating, back pain, malaise and anorexia. It often results in complications and the musculoskeletal system frequently becomes affected [7-9]. The clinical manifestations of human brucellosis depend on various factors including the size of the infectious inoculum, the route of infection, age, duration of the disease and the species of *Brucella spp* infecting the individual [7, 10, 11].

One of the most serious complications of brucellosis is chronic brucellosis [12, 13]. Chronic Brucellosis is defined depending on the duration of the disease, patients were divided into three groups as: acute (<8 weeks), sub-acute (8-52 weeks) and chronic

(>52 weeks) brucellosis. Patients were considered to have relapse/re-infection if symptoms reappeared within a year after the completion of antibrucellar treatment [12-15]. This imposes high costs to patients due to the prolonged course of disease [14]. Today, serology and blood cultures are used to diagnose both classical and chronic brucellosis [14]. However, the slow *in vitro* growth of *Brucella* spp [16] and the lack of specificity of the serology [14], renders management of (chronic) brucellosis and assessment of cure very difficult. Continued antibiotic (re)treatment of brucellosis or not, is a controversial issue [14].

Since cure cannot be reliably assessed using conventional laboratory techniques, more accurate methods are urgently required. Also there is a clear need for more detailed information on chronic brucellosis, along with confirmatory laboratory tests for this version of the disease. In addition there is limited knowledge on the epidemiological and clinical factors predisposing to chronic brucellosis. The aim of the current study is to investigate the prevalence of chronic brucellosis and to examine its epidemiological and clinical features in Central Iran.

MATERIALS AND METHODS

This cross-sectional study was carried out from July 2011 to July 2013 after ethical approval. All patients admitted to the Infectious Diseases Clinic of Valiasr hospital in Arak, Iran, who were diagnosed with chronic brucellosis based on its clinical definition were subjected to present study. Arak is the capital of Markazi province, an endemic area in the Central Iran. Chronic brucellosis was defined for symptomatic patients who upon 12 months after drug treatment still showed a positive serologic test. From all patients included in the study, 5-10 ml of peripheral blood was obtained for serology. Serology tests used and the positive results of each of them for symptomatic patients were defined as follows:

- STAT (Standard Tube Agglutination Test) = 1:160
- Coombs STAT = 1:160
- 2ME (2-mercaptoethanol) = 1:20 [14, 15].

Patients were selected based on the definition of chronic brucellosis, using non-probable sampling method. A questionnaire for collection of demographic, clinical and epidemiologic information was filled out by all

patients. This information included: age, gender, occupation, place of residence, contact with livestock, consumption of dairy products, adherence to antibiotic therapy or treatment compliance and clinical symptoms.

Patient adherence to treatment is defined as the regular usage of antibiotics under the supervision of physicians. Patients were divided into two groups according to their occupation: the first group consisted of patients who had jobs with increased risks for acquisition of brucellosis (e.g. those working with poultry, farmers, butchers, veterinarians and laboratory workers), The second group consisted of patients with jobs unrelated to an enhanced risk for brucellosis. The criteria for inclusion in the study included a positive informed consent, suffering from chronic brucellosis, being between 18-45 years of age, lacking coagulopathy or bleeding issues and a lack of other systemic diseases (e.g. lymphoma, malignancy and autoimmune diseases).

During the study, patients were examined clinically and the clinical findings were recorded in their clinical trial form. Fever was defined when having a body temperature = 38°C. [14]. Peripheral arthritis was diagnosed on the basis of swelling, synovial fluid effusion and limitation of mobility in the joint [17]. Monoarthritis was defined on the basis of involvement of a single peripheral joint and polyarthritis was defined when the simultaneous involvement of several peripheral joints was observed. Sacroilitis was diagnosed using X-ray imaging in supine position (prone) and was confirmed using a bone scan [17]. A diagnosis of epididymo-orchitis was based on the swelling of scrotal skin, orchitis and epididymitis and were confirmed using ultrasound imaging (sonography) [17]. Meningitis was diagnosed when a history of headache was documented in combination with a stiff neck and fever. It was confirmed with STAT and positive 2ME testing of cerebrospinal fluid (CSF) [17].

Endocarditis was diagnosed by examination of ESR (Erythrocyte Sedimentation Rate), anemia and a heart murmur. It was confirmed by the detection of vegetations by echocardiography [17]. Other clinical entities such as hepatosplenomegaly, esophagitis and lymphadenopathy were diagnosed by standard clinical investigations and criteria [14, 17].

Bone marrow aspirates and peripheral blood samples (5-10 ml) were taken and subjected to blood culture and PCR

In addition, results of CBC (Cell Blood Count), ESR (Erythrocyte sedimentation Rate), CRP (C-reactive protein), Cr (Creatinine), AST (aspartate Aminotransferase) and ALT (alanine Aminotransferase) tests were collected.

Blood Culture: Five to ten ml peripheral blood samples were drawn and immediately inoculated under aseptic conditions in broth media (Bloodgrow[®], Medical Wire & Equipment C. Ltd, Corsham, Wiltshire, England) and diphasic blood culture bottles (Hemoline performance diphasique bioMerieux, Marcy l'Etoile, France). Cultures were incubated at 37°C for 30 days in the presence of 5% CO₂ and were periodically checked for growth. Subcultures on blood agar plates were performed in a blinded manner at 10, 20, 30 days, they were recorded as negative after the last negative subculture. *Brucella* spp. were identified using standard methods [18].

DNA Extraction: DNA was extracted from bone marrow aspiration specimens using a commercial purification system (RTP Bacteria DNA Mini Kit, Germany) according to the manufacturer's instructions.

PCR Assay: The *Brucella* genus PCR Kit (Genekam Biotechnology AG, Germany), including the reagents, oligonucleotide primers designed for the direct amplification of the genus *Brucella* and positive control sample was used for the detection of *Brucella* spp. in bone marrow samples. All procedures were carried out according to manufacturers' instructions.

Data were analysed by SPSS software, version 18 (SPSS Inc., Chicago, IL, USA). The Fisher's exact tests (when appropriate) were used for categorical variables and the Chi-square test was used to define correlation between variables. P-values less than 0.05 were considered significant. The study was reviewed and permitted by the Ethics Committee of Arak University of Medical Sciences and a written consent was obtained from all patients. The patients could withdraw from the study at any time. The researchers were committed to the Declaration of Helsinki throughout the research.

RESULTS

Of 1047 patients with brucellosis referred to the clinic of Valiasr Hospital in Arak, 15 patients were diagnosed with chronic brucellosis. Therefore, the prevalence of chronic brucellosis was 1.43%. All these 15 patients were eligible for inclusion and they remained

Table 1: Epidemiological findings in 15 cases of chronic brucellosis

Characteristics of patients	Total No. (%)
Mean age ± S.D.(yr)	43.73±17.22
Gender	
Male	9(60)
Female	6(40)
Job	
Group 1 ^a	8(53)
Group 2 ^b	7(46)
Residence	
Urban	2(13.3)
Rural	13(86.7)
Contact with animal products	
Animal Beef	10(66.6)
Animal skin	7(46.6)
Animal Leather	4(26.6)
Animal Fetus	3(20)
Consumption of Dairy products	
Non-pasteurized ice cream	8(53.3)
Non-pasteurized milk	11(73.3)
Fresh cheese	7(46.6)
Drug Compliance ^c	8(53.3)
Positive	5(33.3)
Negative	10(66.6)

^apatients who had jobs related to brucellosis: poulturer, farmer, butcher, veterinarian and laboratory worker.

^bpatients with job unrelated to the brucellosis . ^c Regular use of medication

in the study up to the end of the study. Of 15 patients with chronic brucellosis, 9 (60%) were male and 6 (40%) were female. As far as their age is concerned, the patients were divided into two groups of = 40 years and <40 years. Eight patients (53.3%) were older than 40 years and 7 (46.6%) were younger than 40 years. The results showed that 8 (53%) patients had jobs related to an elevated risk to acquire brucellosis and 7 (46%) had other jobs. Two (%13.3) patients were city residents and 13 (86.7%) were from rural areas. As far as the chronic exposure to animal products is concerned, long-term contact with meat was found in 10 patients (66.7%) and with regard to the dairy consumption, 11 patients (73.3%) noted the daily use of local milk (Table 1).

In terms of clinical symptoms, all 15 patients complained of weakness and chronic fatigue and the most common clinical finding was peripheral joint arthritis in 12 patients (80%) (Table 2) and 2 (13.3%) versus 9 (60%) patients were diagnosed with monoarthritis and polyarthritis, respectively. Results of the non-specific laboratory tests in patients with chronic brucellosis are shown in Table 3. Of 15 patients with chronic brucellosis, 5 (33,3%) patients were positive in the PCR (Figure 1), of which only one (20%) patient had positive result of blood culture and bone marrow culture

Table 2: Clinical manifestations in 15 cases of chronic brucellosis

Clinical symptoms	Total No. (%)
Weakness	15(100)
Fatigue	15(100)
Sweating	14(93.3)
Arthralgia	12(80)
Anorexia	10(66.6)
Weight Loss	9(60)
Headache	8(53.3)
Mood disturbance	4(26.6)
Nausea	3(20)
Chronic cough	2(13.3)
Abdominal pain	2(13.3)
Neurological symptoms	1(6.7)
Clinical Signs	
Peripheral arthritis	12(80)
Monoarticular arthritis	2(13.3)
Hip	2(13.3)
Polyarthritis	9(60)
Sacroiliac	6(40)
Shoulder	2(13.3)
Knee	7(46.6)
Ankle	1(6.6)
Wrist	4(26.6)
Vertebra	3(20)
Lymphadenopathy	3(20)
Fever ^a	2(13.3)
Hepatomegaly	2(13.3)
Splenomegaly	2(13.3)
Epididymo-orchitis	2(22.2)
Meningitis	0
Bell's palsy	0
Esophagitis	0
Endocarditis	0

^a Fever: >38.8C.



Fig 1: Agarose gel electrophoresis of PCR products. Lane 1-8 bone marrow samples, lane 9 negative and lane 10 positive control

(Table 4). Patients included in present study received antibiotic therapy within eight drug regimens. The results revealed that the most common regimen used by the patients (n=14, 93.3%), is rifampin + doxycycline (Table 5). The clinical and epidemiological information for the 5 brucellosis patients with a positive bone marrow PCR is shown in Table 6. Data analysis did not show a significant correlation between positive bone

Table 3: Nonspecific laboratory test results on 15 cases of chronic brucellosis

Laboratory test	Total No. (%)
WBC^a count	
<4000	1(6.6)
4000-9000	13(86.6)
>9000	1(6.6)
Platelet count	
Normal	15(100)
High	0
Low	0
Anemia^b	
AST^c	
1-32	9(60)
32-50	6(40)
50<	0
ALT^d	
1-50	9(60)
50-100	4(26.7)
100<	1(6.7)
Positive CRP^e	
ESR^f	
20-40 mm/h	7(46.6)
40 mm/h<	1(6.6)
Cr^g	
7-1.4	7(46.6)
1.4 <	1(6.6)

^a white blood cells. ^b male: Hb<13.5 g/dl, female: Hb<12 g/dl. ^c Aspartate Aminotransferase. ^d Alanine Aminotransferase. ^e C-reactive protein. ^f Erythrocyte Sedimentation Rate. ^g Creatinine

Table 4: Specific laboratory test results on 15 cases of chronic brucellosis

Specific laboratory test	Total No. (%)
Blood Culture	
Positive	1(6.6)
Negative	14(93.3)
BM^a culture	
Positive	1(6.6)
Negative	14(93.3)
BM PCR^b	
Positive	5(33.3)
Negative	10(66.6)

^aBone Marrow. ^b Polymerase chain reaction

Table 5: Frequency of antibiotic regimens in 15 patients with chronic brucellosis

antibiotic regimens	Total No. (%)
Regime 1(Rifampin + doxycycline + streptomycin)	12(80)
Regime 2 ((Rifampin + doxycycline)	14(93.3)
Regime 3 (TMP-SMX ^a + rifampin)	4(26.6)
Regime 4 (Ciprofloxacin + rifampin)	1(6.6)
Regime 5 (Ciprofloxacin +doxycycline)	3(20)
Regime 6 (TMP-SMX + rifampin + streptomycin)	1(6.6)
Regime 7 TMP-SMX+ rifampin + doxycycline)	3(20)
Regime 8 (Gentamicin + rifampin + doxycycline)	1(6.6)

^a Trimethoprim-sulfamethoxazole

Table 6: Bone marrow PCR results according to important variables associated with chronic brucellosis infection

BM PCR ^a Results	Residence (n=15)		Job (n=15)		Drug Compliance ^d (n=15)	
	Urban (n=2)	Rural (n=13)	Group 1 ^b (n=13)	Group 2 ^c (n=2)	Positive (n=5)	Negative (n=10)
Positive	0	9(69.3)	4(30.7)	0	1(20)	3(30)
Negative	2(100)	4(30.7)	9(69.2)	2(100)	4(80)	7(70)
Statistical test (PV) ^e	0.36		0.36		0.68	

^a Polymerase chain reaction. ^bpatients who had jobs related to brucellosis: poultterer, farmer, butcher, veterinarian and laboratory worker. ^c patients with job unrelated to the brucellosis. ^d Regular use of medication. ^eP-values less than 0.05 were considered significant in chi-square statistical test

marrow PCR results and important variables related to the brucellosis such as location, occupational exposure and medication compliance (p=0.36, p=0.36, p=0.68, respectively).

DISCUSSION

According to the results of the present study, the prevalence of chronic brucellosis in our region was 1.43%. The prevalence of chronic brucellosis ranged between 5%-19% in different geographical regions [17, 19-21]. The prevalence of brucellosis and different types of its clinical course such as the chronic type of disease, can also be influenced by different environmental and individual factors of each patient, antibiotic regimens and can also get affected by the sample size of the studies, so that the mentioned factors are easily visible when the present study is compared with previous studies.

Based on our study, the percentage of patients with chronic brucellosis having jobs related to this disease (53%) had no difference with the percentage of patients with jobs unrelated to the Brucellosis. In contrast, Hasanjani Roushan [17] found out occupations such as animal husbandry, working in the laboratory and veterinary as the important risk factors and for causing the brucellosis and Nimiri *et al.* [16] showed that 40% of patients had a history of continuous contact with animals. As Brucellosis is basically a zoonotic bacteria, occupations with more contacts between human and animal obviously increase the risk of brucellosis, but this finding, contrary to other studies [16, 17], is not consistent with the results of our study. The main reason for this seems to be deficiency of people with chronic Brucellosis in our two year study.

In contrast to the results for job variable, what was consistent with suffering from chronic brucellosis in most patients of our study was the living place of patients, contact with animal products and the history of constant consumption of unpasteurized dairy products, so that

the present study showed that % 86.7 and % 66.7 of patients are inhabitants of the village and has a history of continuous contact with meat, respectively and almost all patients noted a history of continuous use of non-pasteurized dairy products such as ice cream, milk and cheese. Hasanjani Roushan and Nimiri LF showed that % 60.8 and 44.9% patients with chronic brucellosis were from rural areas and the consumption of unpasteurized dairy products like cheese [17, 18].

In relation to symptoms in patients with brucellosis, most studies had a general evaluation on patients with symptoms and did not report symptoms in patients with certain forms of brucellosis like chronic brucellosis. In a study conducted by Buzgan [19], the most common symptoms of patients were arthralgia (67.1%), fever (10.7 %) and fatigue (62.1%) and the most common clinical findings of patients were hepatomegaly (% 13.6%), splenomegaly (10.7%) and peripheral arthritis (8.6%). Based on the study conducted by Hasanjani Roushan [17], the most common clinical signs and symptoms in patients with Brucellosis were sweating, fever and arthralgia and the most common clinical findings were peripheral arthritis (with the preference of monoarthritis) (9.2%) and splenomegaly (5.8%).

Khateeb [20] showed that the most clinical symptoms of patients with brucellosis were arthralgia, fever and sweating, respectively. Geyik *et al.* [22] and Tasova *et al.* [10], each in a separate study, reported the incidence of peripheral arthritis in patients with brucellosis as 30.7% and 19%, respectively. According to these studies, apart from clinical form of brucellosis and some slight differences in the prevalence of clinical symptoms and findings and also taking into account the different sample size of patients in different studies, the results of studies are similar with each other and in accordance with present study. Hence, it can be said that the most common clinical symptoms in patients with chronic brucellosis are fever and arthralgia and the most common clinical finding was associated with the peripheral arthritis.

Prevalence and pattern of musculoskeletal system involvement in patients with brucellosis depends on the bacterial agent that causes disease and the duration of disease [17]. Joint infection is a common side effect of brucellosis and is considered as one of the common causes of septic arthritis in endemic areas [17]. According to our study, the most commonly involved peripheral joints were knee and sacroiliac joints. Hasanjani Roushan [17] reported sacroiliac, hip and knee joints and Khateb [20] reported sacroiliac joints and knees as the most commonly involved joints in patients with peripheral brucellosis.

Geyik [22] showed that sacroiliac joint involvement with the prevalence of 55% is the most commonly involved joint in patients with brucellosis. Based on the results of our study and similar studies, it seems that sacroiliac joint involvement is the most common clinical finding in the involvement of musculoskeletal in patients with brucellosis.

Epididymo-orchitis, common clinical symptom in patients with brucellosis which, based on the studies, could involve patients by the rate of about 17.5% [17]. In our study, % 2.22 of patients with chronic brucellosis suffered from epididymo-orchitis. This finding, compared with the findings of other studies in this field which reported the range of incidence of epididymo-orchitisas % 1.6 to 17% [23-26] was found different and higher.

Infective endocarditis is one of the devastating complications of brucellosis. In our study, the incidence of this complication was 0%; while Hasanjani Roushan [17] obtained the incidence of endocarditis among 469 patients with Brucellosis as % 0.5. Memish, [25] and Namidura [26], reported the incidence of infective endocarditis among 530 patients with Brucellosis as 1.5%. Although the incidence of endocarditis in our study differs from previous studies (which clearly could be due to differences in sample size), but in general it can be concluded that the incidence of endocarditis in patients with brucellosis has very low frequency, compared with other clinical findings. Blood disorders such as anemia, leukopenia and thrombocytopenia are common in patients with brucellosis [17].

In our study, leukocytosis, thrombocytosis and anemia, were seen in %6.6, % 0, % 13.3 of the patients, respectively. The prevalence of above-mentioned had little frequency compared with the studies conducted by Hasanjani Roushan [17], Namidura [26], Geyik [22] and Akdeniz [27].

The antibiotic regimen is an appropriate choice for the therapy of human Brucellosis in many cases [28]. Although a number of studies have suggested some risk

factors for the treatment failure, relapse and chronic infection[29, 30], but still the best antibiotic regimen for the treatment of patients with brucellosis has not been introduced [28]. The results of our study showed that the most common regimen used by patients with chronic brucellosis with the rate of % 93.3, is doxycycline + rifampin (DR) regimen. In other words, DR antibiotic regimen has the most association with susceptibility to chronic Brucellosis. Alavi SM, et al [28] studied the frequency of treatment failure and relapse of Brucellosis following different antibiotic treatments. The results of this study showed that streptomycin + doxycycline (SD), doxycycline + rifampin (DR) and doxycycline + gentamicin (DG) antibiotic regimens are associated with % 7.4 failure and %4.8 relapse, % 7.8 failure and %10.7 relapse and % 5.2% failure and 5.9% relapse, respectively in patients with brucellosis. Based on the results of our study and the study of Alavi SM [28] it seems that the use of antibiotic regimen of doxycycline + rifampin (DR) is more associated with the failure, relapse and chronic Brucellosis compared with other regimens of treatment.

The main objective of this study was to answer the question of whether the patients diagnosed with chronic Brucellosis is really suffering from a chronic form of this disease or they are suffered from other infectious and non- infectious diseases with clinical symptoms similar to brucellosis? In other words, the main objective of the present study was to prove the diagnosis of chronic Brucellosis. So, the present study showed that 6.6%, % 6.6 and % 33.3 of patients had positive results of blood cultures, cultures of bone marrow and bone marrow PCR sample, respectively and only 20% of patients with positive bone marrow PCR, had positive blood culture and bone marrow culture results. Despite negative blood cultures in four chronic Brucellosis patients, PCR assay was able to detect bacterial DNA in bone marrow samples. As we know, bacterial DNA will be destroyed in the body through nuclease enzymes activity in body fluids. Hence, it can be suggested that these bacteria might remain alive and infective but in a latent form controlled by the host immune system.

In a study on 140 patients with chronic brucellosis, Buzgan T [19] showed that %3.6 and %1.4 of patients had a positive blood culture and bone marrow culture, respectively. Nimiri LF [18] showed that among 165 patients with symptomatic positive serology of Brucellosis, %72.7 and 12% of patients had positive peripheral blood PCR and blood culture results, respectively and only 12% of patients had positive test results of blood cultures, peripheral blood PCR and serology. Aygen B [21] reported the prevalence of

positive blood culture among seropositive patients suffering from Brucellosis as 45%. According to the results of this study and other studies and the fact that all the cases in our study were seropositive, it is clear that the blood culture, bone marrow culture and bone marrow PCR is not always positive where serology is positive and all PCR -positive cases are not associated with positive blood culture and bone marrow culture. As the PCR has the sensitivity and specificity of 100% and a positive predictive value of % 85.7 [14] to Brucellosis diagnosis and the positive results of this test confirms the bacterial presence in the body.

Our study showed that the negative results of blood culture do not reject Brucellosis infection, because in this study, of five patients with positive PCR, only one patient had a positive blood culture. Therefore, considering the above cases, it is suggested that in the cases of suspected infection with brucellosis, relapses [14], especially in chronic cases of this disease, we shall not be satisfied with only using serology tests or even tissue culture; and beside these tests and follow-ups of the patients further microbiological methods such as blood or bone marrow PCR should be used.

CONCLUSIONS

Results of the study showed that epidemiological and clinical factors, such as living in the village, contact with meat, constant consumption of local milk and rifampin + doxycycline antibiotic regimen, are more associated with susceptibility to chronic brucellosis. The results also indicated that for the diagnosis and follow-up of patients with Brucellosis, especially in chronic cases of the disease, complementary use of microbiological diagnostic techniques such as PCR along with blood culture and serology methods can be useful in clinical dealing with the patients with chronic Brucellosis.

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Conflict of Interest: Nothing to declared

Authors Contribution: Dr Nader Zarinfar has been involved in study design, bone marrow aspiration and writing the draft. Dr Samaneh Saburi contributed in sample

collection and was responsible for serological tests, blood and bone marrow cultures and editing the draft. Dr Ehsanollah Ghaznavi-rad was in charge for data analysis, PCR assay and editing the draft.

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