DISTURBED GUT MICROBIOTA EXPLAINS PERSISTENT ABDOMINAL PAIN AFTER A MAJOR ENVIRONMENTAL DISASTER

BY

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Dr Nurfadhilah Yusof

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ABSTRAK

Latar Belakang:

Adalah tidak diketahui jika terdapat hubungan kait antara sakit perut dengan kekurangan amalan penggunaan air yang bersih, sanitasi dan amalan kebersihan (*WaSH*) dan sekiranya penumbuhan bacteria usus kecil (*SIBO*) atau usus *dysbiosis* saling berkait dengan sakit perut selepas banjir besar.

Kaedah:

Simptom gejala sakit perut, kualiti hidup, gangguan psikologi, amalan kebersihan (WaSH), *SIBO* dan sampel najis bagi penjujukan berskala besar dinilai pada mangsa banjir.

Keputusan:

Seramai 211 peserta (min umur 54.5 tahun, perempuan 71%), 37.9% mempunyai sakit perut. Kekurangan amalan *WaSH* semasa banjir dan sindrom usus (IBS) dikaitkan dengan sakit perut (kedua-dua P <0.04). Mereka yang mempunyai sakit perut mengalami kualiti hidup lebih teruk dan cenderung mengalami kebimbangan yang berlebihan (kedua-dua P <0.05). SIBO dikaitkan dengan sakit perut (P = 0.01), amalan penggunaan air yang kurang baik semasa banjir (P = 0.04) dan fungsi fizikal yang terhad (P = 0.02). Menggunakan analisis kaedah linear diskriminasi, usus *dysbiosis* usus diperhatikan pada mereka yang mempunyai tahap kebimbangan (*Bacteroidetes* dan *Proteobacteria*, saiz kesan 4.8), sakit perut (*Fusobacteria, Staphylococcus, Megamonas* dan *Plesiomonas*, saiz kesan 4.0) dan IBS (*Plesiomonas* dan *Trabulsiella*, saiz kesan 3.0).

Kesimpulan:

Microbiota usus terganggu kerana organisma alam semulajadi yang diperolehi boleh bersaling kait dengan sakit perut berterusan selepas bencana alam besar. Ini didapati berkait juga dengan kekurangan amalan *WaSH* .

ABSTRACT

Background:

It is unknown if there is association between abdominal pain and poor water, sanitation and hygiene (WaSH) practices and if small intestinal bacterial overgrowth (SIBO) and/or gut dysbiosis explains abdominal pain after a major flood.

Methods:

Abdominal symptoms, quality of life, psychological disturbances, WaSH practices, SIBO and stools for high-throughput sequencing were assessed in flood victims.

Results:

Of 211 participants (mean age 54.5 years, females 71%), 37.9% had abdominal pain. Poor WaSH practices during flood and irritable bowel syndrome (IBS) were associated with abdominal pain (both $P < 0.04$). Those with abdominal pain experienced worse quality of life and more anxiety (both $P < 0.05$). SIBO was associated with abdominal pain ($P = 0.01$), poor water practices during flood ($P = 0.04$) and limited physical functioning ($P = 0.02$). Using linear discriminant analysis effect size method, gut dysbiosis was observed in those with anxiety (*Bacteroidetes* and *Proteobacteria*, effect size 4.8), abdominal pain (*Fusobacteria, Staphylococcus, Megamonas* and *Plesiomonas*, effect size 4.0) and IBS (*Plesiomonas* and *Trabulsiella*, effect size 3.0).

Conclusions:

Disturbed gut microbiota because of environmentally-derived organisms may explain persistent abdominal pain after a major environmental disaster in the presence of poor WaSH practices.

CHAPTER 1

LITERATURE REVIEW

1.1 LITERATURE REVIEW

The massive flood disaster in December 2014 that occured in the north-eastern state of Peninsular Malaysia has affected over 230,000 people, leaving 2000 homeless and approximately 11 dead. Besides significant financial loss and psychological trauma, the flood victims have to endure increased risks from water-borne and other communicable diseases[1]. Our preliminary community survey of one massive flood-affected area (district of Tumpat, Kelantan) showed that approximately 30-40% of the population had reported abdominal symptoms during the flood (unpublished data). These victims are at risk for developing post-infectious irritable bowel syndrome (IBS) 3 to 12 months later[2]. One possible reason for persistence of abdominal discomfort is poor water, sanitation and hygiene (WaSH) practices by the flood victims during and after flood and their relationship has not been clearly studied previously. The flood lasted for about a week, and not all flood victims' had access to clean drinking water, and also water to wash and prepare food during that time. Ingestion of fecal pathogens in the flood water may have resulted in small intestinal bacterial overgrowth (SIBO) and consequently enteropathic changes in the small bowel and then development of chronic abdominal discomfort [3]. Therefore, our study objectives were firstly, to establish the connection between chronic abdominal discomfort and poor WaSH practices during flood, and secondly, to determine if SIBO plays a role in the causation of abdominal discomfort in a flood-affected community with poor WaSH practices.

CHAPTER 2

OBJECTIVES OF THE STUDY

2.1 To establish the connection between chronic abdominal discomfort and poor WaSH practices during flood and to determine if SIBO and disturbance in gut microbiota play a role in the causation of abdominal discomfort in a flood-affected community with poor WaSH practices.

2.2 Specific Objectives

1. To determine the prevalence of abdominal complaints, WaSH practices, psychological disturbance and assess quality of life in flood victims

2. To determine the association between WaSH practice in flood victims with vs. without abdominal complaints

3. To determine the association between SIBO in flood victims with vs. without abdominal complaints

4. To determine the association between gut microbiota profiling using pyrosequencing in flood victims with vs. without abdominal complaints

CHAPTER 3

MANUSCRIPT

3.1 **Disturbed gut microbiota explains persistent abdominal pain after a major environmental disaster**

Running title: Dysbiosis and abdominal pain after flood

Journal: Epidemiology & Infection

ABSTRACT

It is unknown if there is association between abdominal pain and poor water, sanitation and hygiene (WaSH) practices and if small intestinal bacterial overgrowth (SIBO) and/or gut dysbiosis explains abdominal pain after a major flood. Abdominal symptoms, quality of life, psychological disturbances, WaSH practices, SIBO and stools for high-throughput sequencing were assessed in flood victims. Of 211 participants (mean age 54.5 years, females 71%), 37.9% had abdominal pain. Poor WaSH practices during flood and irritable bowel syndrome (IBS) were associated with abdominal pain (both $P < 0.04$). Those with abdominal pain experienced worse quality of life and more anxiety (both $P < 0.05$). SIBO was associated with abdominal pain ($P = 0.01$), poor water practices during flood ($P = 0.04$) and limited physical functioning ($P = 0.02$). Using linear discriminant analysis effect size method, gut dysbiosis was observed in those with anxiety (*Bacteroidetes* and *Proteobacteria*, effect size 4.8), abdominal pain (*Fusobacteria, Staphylococcus, Megamonas* and *Plesiomonas*, effect size 4.0) and IBS (*Plesiomonas* and *Trabulsiella*, effect size 3.0). Disturbed gut microbiota because of environmentally-derived organisms may explain persistent abdominal pain after a major environmental disaster in the presence of poor WaSH practices.

Keywords: flood; Malaysia; water, sanitation and hygiene practices; abdominal pain; dysbiosis; small intestinal bacterial overgrowth

INTRODUCTION

In December 2014, a massive river-flood disaster affected 230,000 people in the north-eastern region of Peninsular Malaysia, leaving 2,000 homeless and approximately 21 dead (Fig. 1). Besides significant financial loss and psychological trauma, the victims endure increased risks from water-borne communicable diseases [1].Children are most affected by diarrhoeal diseases but significant symptoms and psychological morbidity may be worse among adults. Many victims had poor water, sanitation and hygiene (WaSH) practices during the flood and post-flood period. They had limited access to clean water for drinking and preparing food as well as limited access to clean toilet facilities. It is postulated that ingestion of faecal pathogens in contaminated flood water because of poor WaSH practices may cause small intestinal bacterial overgrowth (SIBO) and dysbiosis in the gut [3, 4]. As a result, adult flood victims may develop persistent abdominal pain akin to post-infectious irritable bowel syndrome (IBS) 3 to 12 months later [2]. Therefore, our study objectives were to establish the association between persistent abdominal pain and quality of life, psychological disturbance and poor WaSH practices in adults during flood, to determine if SIBO was associated with abdominal pain in a flood-affected adult community with poor WaSH practices, and finally, to describe the gut microbial taxa in stool of this flood-affected adult community with abdominal pain.

METHODS

Study design and population

The present study was a cross-sectional study involving adult participants from two villages located approximately 25 km from the city of Kota Bharu, in the north-eastern region of Peninsular Malaysia (Fig. 1). The two villages, namely Kok Keli and Kok Pasir have a population estimate of 3,700 and both villages were badly affected during the flood in December 2014. Each flood-affected household in these villages was approached sequentially between August 2015 and November 2015 based upon a list provided by the Village head. After adult participants in these households provided their informed consent and were surveyed for symptoms, participants were asked to complete other questionnaires regarding quality of life, psychological disturbance and WaSH practices. In addition, participants were asked to provide a sample for hydrogen breath testing and a stool sample for molecular analysis. Inclusion criteria consisted of participants aged 18 and above and affected by the river-flood in December 2014. Exclusion criteria included history of abdominal symptoms prior to the river-flood, history of antibiotics or probiotics 3 months prior to and after flood, inability to provide breath and stool samples, chronic medical illnesses (especially neurological diseases e.g. strokes and cancers) and previous abdominal surgeries and psychiatric illnesses. This study was approved by the Universiti Sains Malaysia (USM) Human Research Ethics Committee (USM/JEPeM/15040133) which complies with acceptable international standards including the Declaration of Helsinki.

Assessment of symptoms, quality of life and psychological distress

Participants were asked if they had new onset abdominal pain that persisted for 6 months after the flood. In order to exclude pre-existing functional gastrointestinal (GI) disorders, participants were asked for any prior history of abdominal symptoms and also whether they had taken any medications to relieve abdominal symptoms. Demographic data including age, gender, marital status and educational status were also captured. Questionnaires administered included the Malay version of Rome III Questionnaires for IBS, functional dyspepsia (FD) and also the gastroesophageal reflux disease questionnaire (GERDQ) [5,6, 7]. A diagnosis of IBS, FD, and FC were made based on previously published Rome III criteria [8].For quality of life (QOL) assessment, the validated Malay version of SF-36 was administered; this questionnaire consists of 36-items that measured eight domains, namely physical (10-item) and social functioning (2-item), role limitations in physical health (4-item), role limitations due to emotional problems (3-item), energy (4-item), mental well-being (5-item), bodily pain (2-item) and general health perception (5-item) [9]. Each domain of SF-36 has a score range of 0-100, with lower score signifying worse QOL. For assessment of anxiety and depression, the Malay version of the Hospital Anxiety and Depression Scale (HADS) was administered; this questionnaire consists of 14-items with four-point Likert responses. Each domain of HADS is scored as a continuous variable [10].

Assessment of water, sanitation and hygiene (WaSH) practices

According to WHO/UNICEF, poor water practice includes the use of water from contaminated sources; poor sanitation practice means no clean toilet facility; and poor hygiene practice includes washing hands with no soap, no hand-washing or no bathing facilities in the house [11]. Above is the basis for a specifically developed questionnaire to assess WaSH practices of victims during the flood. A group of experts (physicians and public health experts) were responsible to draft the 10-item questionnaire based on their WaSH experiences with flood victims but also with literature review. The WaSH practice questionnaire consists of three domains, namely water (4-item), sanitation (3-item) and hygiene (3-item). Responses were in 5-point Likert scale (Supplementary Material 1). Scores for each domain and a mean total score of all domains of WaSH were calculated as continuous variables, higher scores signified poorer WaSH practices.

Breath-testing for small intestinal bacterial overgrowth (SIBO)

SIBO is postulated to be associated with post-flood symptoms and hydrogen breath test is a suitable non-invasive method to diagnose SIBO.Agreed participants would exhale end-expiratory breath samples into a collection bag at baseline. Then they were asked to drink 75 g of glucose in cold water [12]. At intervals of 15 mins for the next 2 hours, breath samples would be collected and symptoms recorded [12]. The breath samples were brought back to the hospital and tested within 24 – 48 hours. Forty mL of exhaled breath would be syringed into the machine (Quintron, Milwaukee, US) and levels of H_2 and CH_4 (in parts per million or ppm) determined. For a positive test, the following criteria were applied: a rise in H_2 value ($\geq$ 20 ppm) or CH_4 values ($\geq$ 10 ppm) above fasting baseline value or a sustained rise in H_2 or CH_4 of 5 ppm over 3

consecutive breath samples [12].A rise in breath values as above and reproduction of symptoms were required to diagnose SIBO.

Assessment of fecal specimen

Fecal specimens defecated on a rice paper in lavatory bowl were collected in a sterile container. After that, two spatula portions of the fecal specimens were transferred into a sterile fecal collection tube, and capped tightly. The collection tube was pre-filled with 2 – 4 mL of RNAlater® stabilization solution (Thermo Fisher Scientific, USA) and four glass beads. The tube was shaken vigorously for 10 s to suspend the feces in the solution. Upon transfer to the lab, total DNA from 20 mg of fecal samples, which were precipitated by centrifugation, was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Purified DNA was suspended in 2000 μl of Tris-EDTA buffer (pH 8.0). Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA geneV3-V4 region was performed with the TaKaRa Ex Taq HS Kit (TaKaRa Bio, Shiga, Japan) with the primer sets Tru357F (5'-CGCTCTTCCGATCTCTGTACGGRAGGCAGCAG-3') and Tru806R (5'-CGCTCTTCCGATCTGACGGACTACHVGGGTWTCTAAT-3'). The sample of DNA was then analysed using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the method by Odamaki et al. [13]. Trimmed reads <150 bp in length with a mean quality score < 25 were removed. The fastq-join script in EA-Utils (version 1.1.2-537) was used to combine the reads that passed the quality filters. For the taxonomic analysis, the sequences were analysed by the QIIME software package version 1.8.9 (http://qiime.org/). The potential chimeric sequences were removed by UCHIME, which was assigned to the open-reference

operational taxonomic units (OTUs). The sequences were taxonomically classified by the Greengenes reference database [13].

Statistical analysis

Continuous variables were presented in mean ± standard error of mean (SEM) unless otherwise mentioned. Analysis was performed using Chi-square or Fisher-exact for categorical data and t-test for continuous data. Binary logistic regression analysis (odds ratio [OR] and 95% confidence interval [CI]) was used to test for factors associated with abdominal pain, poor WaSH practices during flood and SIBO, respectively.Principal component analysis (PCoA) based on Jensen-Shannon divergence (JSD) was performed using R version 3.2.4and linear discriminant analysis (LDA) effect size or LEfSe method for microbial taxa composition were performed on the Galaxy web site (https://huttenhower.sph.harvard.edu/galaxy) [14,15].A *P*-value <0.05 was considered as significant.

RESULTS

Characteristics of study participants

Out of 272 screened villagers, 211 participants (mean age 54.5 ± 1.0 years, females 71%) met the study criteria and agreed to participate. Characteristics of participants are shown in **Table 1**. Abdominal pain that persisted following flood was seen in 37.9% or 80 participants. Of those with abdominal pain, 45% or 36 participants had IBS with diarrhea and/or mixed-type (present in 33.8%), reporting greater IBS severity with a mean score of 107.4. Those with pain were also

more likely to have FD and a higher GERDQ score (all *P* values < 0.05) than those reporting no pain.

Relationship between pain, QOL, psychological morbidity and WaSH practices

General health (65.6 ± 16.0) and physical functioning (65.7 ± 28.4) domains of SF-36 scored the lowest among all flood-affected participants. In those with pain vs. no pain, emotional problems (*P* = 0.04) and bodily pain (*P* = 0.009) scored significantly lower(**Table 1**). In addition, those with pain vs. without pain had significantly higher anxiety scores (4.0 ± 0.4 vs. 2.9 ± 0.4, *P* = 0.04). Abdominal pain was significantly associated with increased total scores of all three domains of WaSH practices (P=0.04). Among the three domains, only poor water practices (including poor quality, colour and taste of water supply during flood) were significantly associated with pain (P=0.005). With logistic regression analysis, persistent abdominal pain was associated with a greater total score of WaSH during flood (OR 1.11, 95% CI 1.00; 1.24, *P* = 0.04) and IBS (OR 9.53, 95% CI: 3.51; 25.86, *P* < 0.001).

SIBO is associated with abdominal pain during flood

Although 211 participants completed the questionnaires only 135 consented for subsequent breath testing for SIBO. Results of these 135 participants were subsequently analysed and reported for association between SIBO and pain. Of the 135 participants (mean age 55.6 ± 1.3 years, females 98 or 72.6%), 31.9% reported abdominal pain and 12.6% were SIBO positive. Of participants positive vs. negative for SIBO, persistent abdominal pain was more likely (47.1% vs. 15.3%, *P* = 0.02) and they also reported worse water practices during flood (9.5 ± 2.0 vs. 7.8

± 0.2, *P* = 0.04). Besides that, those with vs. without SIBO were more likely to have impaired physical functioning (51.2 ± 8.3 vs. 67.8 ± 2.5, *P* = 0.02) and social functioning (70.7 ± 6.8 vs. 84.0 ± 1.7, *P* = 0.01). Those with vs. without SIBO were also more anxious (5.2 ± 1.3 vs. 3.1 ± 0.2, *P*= 0.01). With multiple logistic regression analysis (backward-LR), abdominal pain (OR 4.94, 95% CI 1.47; 16.55, *P* = 0.01), poor water practices during flood (OR 1.14, 95% CI 1.01; 1.30, *P* = 0.04) and limitation in physical functioning (OR 0.98, 95% CI 0.96; 1.00, *P* = 0.02) were significantly associated with positive SIBO.

Gut dysbiosis is associated with psychological disturbance and abdominal pain

Of 135 participants consented for breath testing, 73 agreed to give their stools for high throughput sequencing and subsequent analysis. Of 73 participants (mean age 55.8 ± 1.6 years, females 53 or 72.6%), 21.9% had abdominal pain and 17.8% had IBS. The predominant phyla in all 73 participants were Bacteroidetes (37.1%), Firmicutes (24.6%) and Proteobacteria (8.4%). With PCoA of gut microbiota composition, two different clusters based on HADS anxiety scores instead of other scores (SF-36 and HADS depression scores) were observed (*P*< 0.05) (**Figure 2**).

The most differentially abundant bacterial taxa observed in the cluster with a higher anxiety score (mean score 4.0, cluster 1) were the phyla *Bacteroidetes* (including the genus *Prevotella*) and *Proteobacteria* with effect size of 4.8. The Shannon Index was significantly lower in the cluster with more anxiety (mean score 4.0, cluster 1) than with less anxiety (mean score 2.0, cluster 2) (4.8 vs. 5.5, P < 0.001).

Figure 3 (A and B) shows the taxonomic representation and histogram of LDA scores of participants with abdominal pain. The phylum *Fusobacteria* was the most abundant with LDA effect size of 4.0. Others included the genus *Staphylococcus, Megamonas* and *Plesiomonas*. **Figure 3 (C and D)** shows the taxonomic representation and histogram of LDA scores of participants with and without IBS.The most differentially abundant bacteria taxa observed in those with IBS was the genus *Plesiomonas* and *Trabulsiella* with effect size approaching 3.0.

DISCUSSION

Our study provides evidence that persistent abdominal pain is common (37.9% of studied population) over six months following a flood disaster, and the pain was due to IBS in more than two-thirds of flood-affected adults. For comparison, 36% of IBS cases were reported over 24 months in the Walkerton outbreak of *Escherichia coli* and *Campylobacter jejuni*found incontaminated water [16].Flood-affected adults with abdominal pain due to IBS reported a higher IBS severity score and a third were of diarrheal and/or mixed-type.A meta-analysis by Halvorson et al. of eight studies reported a 7-fold IBS risk following acute gastroenteritis [17], and in our study, the IBS risk following flood was 9.5-fold.Because of similar pathophysiology affecting the gut-brain axis, other functional GI disorders including FD and GERD were also reported among flood victims who developed IBS[18].

Patients with severe IBS especially in the presence of abdominal pain have a poorer health-related QOL [19, 20, 21]. The same may occur with flood victims in our study and SF-36 allows us to assess both physical and mental functioning of these victims. Emotional problems and bodily pain were found to be significantly associated with abdominal pain after flood (Table