Quantifying Impact of Confounding Variables in *Blastocystis*/IBS Association Studies

Ken Boorom
Blastocystis Research Foundation
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About BRF / Your Presenter

• Blastocystis Research Foundation est. 2006 in Oregon, USA
• Contact: kboorom@bhomcenter.org / www.bhomcenter.org
• Supported by researcher volunteer time + public donations
• How do we get highest value from research?
• What research topics are the most relevant?
• Which ideas are most likely to be successful?
• How do we teach researchers to get IRB approval?
IBS Parameters

- High morbidity, low mortality (although associated with 3x suicide rate increase)
- Physicians, researchers, and research groups themselves are often patients (41% of medical professionals in Pakistan)
- QOL scores similar to severe cardiovascular disease

_Blastocystis_ Parameters

- All major subtypes (ST1-4) in symptomatic + asymptomatic patients
- ~50% asymptomatic in one study, but depends on ST and patient group
- Spontaneous clearance rate 22% in symptomatic (Vanhellemond, 2012)
- Metronidazole raises this to 36%, paromycin to 77%
- In chronic cases, infection length is indefinite. MZ efficacy=0%
- (Probably most important) Only highly prevalent GI protozoal infection with no reliable eradicative therapy
Blastocystis and IBS – Brief History

• 1986 – Markell et al - *Blastocystis* patients actually have IBS
• 1997 – Hussain, et al - *Blastocystis* as the cause of IBS (IgG resp)
• 1991 – 1/3 US deployed military return from Gulf War with IBS
• 1999 – Giacommetti, et al - *Blastocystis* as cause of IBS
• 2005 – Multiple types of Blastocystis – are some non-pathogenic?
• 2007 – Stark, et al– Don’t diagnose IBS in *Blastocystis* patients
• 2008 – Jones, et al - first US study on *Blastocystis*
• 2008 – Boorom, et al - First major review, *Blastocystis* and IBS
• 2010 – IBS/Blastocystis link not significant in Thailand (10% vs 16.7%)
• 2010++ - Yakoob, et al – *Blastocystis*, IBS, medical students + workers
• 2012 – Jimenez-Gonzalez, et al – Link is significant in Mexico
• 2012 – Olivio-Diaz – First host genetic susceptibility polymorphisms
Fig. 7.1  Cumulative NIH indexed studies for three common intestinal protists. In terms of the total number of studies, *Blastocystis* spp. lags *Giardia intestinalis* by 30 years, and *Cryptosporidium* spp. by 20 years. Most of the controversy concerning *Blastocystis* infection occurred between 1986 and 1993, when there were very few studies on the infection available.
Fig. 7.2 Number of new NIH indexed studies published each year for three common intestinal Protists. The rate of publication for *G. intestinalis* and *Cryptosporidium* spp. is about 5–7 times higher than that for *Blastocystis*.
Fig. 7.4 Analysis of NIH-Indexed *Blastocystis* Studies as of January 2008. Qualifying studies were those related to infection in immunocompetent individuals where the researcher reported a finding concerning pathogenicity based on a scientific investigation.
Review

Oh my aching gut: irritable bowel syndrome, Blastocystis, and asymptomatic infection

Kenneth F Boorom*1, Huw Smith2, Laila Nimri3, Eric Viscogliosi4, Gregory Spanakos5,Unaiza Parkar6, Lan-Hua Li7, Xiao-Nong Zhou8, Ülgen Z Ok9, Saovanee Leelayoova10 and Morris S Jones11

Address: 1Blastocystis Research Foundation, 5060 SW Philemuth Blvd, #202, Corvallis, OR 97333, USA, 2Scottish Parasite Diagnostic Laboratory, Stobhill Hospital, Glasgow, G21 3UW, UK, 3Department of Medical Laboratory Sciences, Jordan University of Science & Technology, Irbid, Jordan 22110 (currently at Center for Disease Control, Atlanta, CA, USA), 4Unite' Inserm U547, Institut Pasteur, 1 Rue du Professeury Calmette, BP 245, 59019 Lille Cedex, France, 5Department of Parasitology, Entomology and Tropical Diseases, National School of Public Health, 196 Alexandras Ave, 11521 Athens, Greece, 6WHO Collaborating Centre for the Molecular Epidemiology of Parasitic Infections and the State Agricultural Biotechnology Centre, School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Western Australia 6150, Australia, 7Department of Preventive Medicine, Weifang Medical University, 288 Shengli East Street, Shandong, Weifang, 261042, People's Republic of China, 8National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, 200025, People's Republic of China, 9Department of Parasitology, Faculty of Medicine, Celal Bayar University, Manisa, Turkey, 10Department of Parasitology Phramongkutklao College of Medicine, Ratchathewi, Bangkok 10400, Thailand and 11Clinical Investigation Facility, David Grant USAF Medical Center, 101 Bodin Circle, Travis AFB, CA 94535, USA

Email: Kenneth F Boorom* - director@bhomcenter.org; Huw Smith - Huw.Smith@NorthGlasgow.Scot.NHS.UK; Laila Nimri - nimri@just.edu.jo; Eric Viscogliosi - eric.viscogliosi@pasteur-lille.fr; Gregory Spanakos - grspan@yahoo.com; Unaiza Parkar - unaiza@inet.net.au; Lan-Hua Li - orchid8@sina.com; Xiao-Nong Zhou - ipdzhoux@sh163.net; Ülgen Z Ok - okulgen@superonline.com; Saovanee Leelayoova - s_leelayoova@scientist.com; Morris S Jones - Morris.Jones@travis.af.mil

*Corresponding author


“IBS is the only functional GI disorder where a protozoal infection has been found...
In almost half of the diagnosed cases…”
Blastocystis spp. FAQs

What is Blastocystis spp.?
Blastocystis is a common microscopic organism that inhabits the intestine and is found throughout the world. A full understanding of the biology of Blastocystis and its relationship to other organisms is not clear, but is an active area of research. Infection with Blastocystis is called blastocystosis.

What are the symptoms of infection with Blastocystis?
Watery or loose stools, diarrhea, abdominal pain, anal itching, weight loss, constipation, and excess gas have all been reported in persons with Blastocystis infection. Many people have no symptoms at all. The organism can be found in both well and sick persons.

How long will I be infected?
Blastocystis can remain in the intestine for weeks, months, or years.

Is Blastocystis spp. the cause of my symptoms?
The role of Blastocystis in causing disease is controversial among experts. Some types of Blastocystis may be more likely to be associated with symptoms. Finding Blastocystis in stool samples should be followed up with a careful search for other possible causes of your symptoms.

Is having blastocystosis common?
Yes. In fact, many people have Blastocystis in their intestine, some without ever having symptoms.
Patient Statements

Contracted in Sudan

Saturday, February 6, 2010, 9:23 pm [EST]


My story is sadly very familiar to so many others on this site. This disease has had a considerable impact on my life, although I feel fortunate to have managed to pursue my career, and start a family.

I'm contacting you because none of the treatments I've been tried have helped (I've tried all the main treatment combinations, both conventional and natural) except for my most recent round of medication.

In December, I went to a primary care physician to test my blood sugar level as a friend had mentioned that some of my symptoms suggested diabetes. A native of Ghana, he looked at my history and declared that I had chronic Malaria. (I did have a history of Malaria in Sudan).

He advised me to take a 20 day course of Lariam at 250 mg a day. (The standard dose for malaria prevention is 250 mg a week.)
Patient Statements

J in California
Green Juice
“4 years ago I went to buy a green juice….my life has been hell: I can not sleep because of a heat feeling on my shoulder area and all over my body, colitis, leaky gut, blooded, feeling suicidal, restless, fog like feeling, etc. Before this I had a normal life worked out 4 to five times a week but now I'm miserable.
—J in California

Received 3/28/2009
3.5 months of chronic diarrhea, skin eruptions and nothing fazes it. Tell me there's *something* that can at least end the D!

B on East Coast
July 2007
I have spent my married reproductive years with this beast and since I can not eat did not think I could make a healthy baby. But as 40 is here I am not willing to give up on the idea of a child altogether because this parasite stole the option for us to try and have a baby.

B in California
Widespread Pain
“Our main symptoms have been complete loss of appetite, feeling very unwell (stomach feels full and heavy) after eating anything, subsequent weight loss and low levels of energy, fatigued etc, some nausea. (no real pain, very little diarrhea, no vomiting)…. Chronic fatigue, confusion,


Mono-infections with *Blastocystis* spp, *Cryptosporidium* spp., and *Entamoeba histolytica* all present this pattern. *Giardia intestinalis* presents a similar pattern without constipation. Viral/bacterial infections present a different pattern.

*Blastocystis* spp., *Cryptosporidium* spp., and *Entamoeba histolytica* exhibit similar symptomatic and epidemiological patterns in healthcare-seeking patients in Karachi.
## “Fingerprinting” Infections

<table>
<thead>
<tr>
<th>Symptom</th>
<th>All Patients (n=339)</th>
<th>Patients with any parasite present (n=163)</th>
<th>Patients with <em>Blastocystis</em> spp. Present (n=59)</th>
<th>Patients with <em>Blastocystis spp.</em> Mono-infection (n=34)</th>
<th>Patients with <em>Cryptosporidium</em> spp. present (n=37)</th>
<th>Patients with <em>E. histolytica/dispar</em> present (n=46)</th>
<th>Patients with <em>Giardia intestinalis</em> present (n=24)</th>
<th>Patients with no Parasite Found (n=176)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal Pain</td>
<td>71% (240/339)</td>
<td>48% (78/163)</td>
<td>46% (27/59)</td>
<td>38% (13/34)</td>
<td>49% (18/37)</td>
<td>46% (21/46)</td>
<td>58% (14/24)</td>
<td>92% (162/176)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>35% (120/339)</td>
<td>65% (106/163)</td>
<td>64% (38/59)</td>
<td>59% (20/34)</td>
<td>54% (20/37)</td>
<td>63% (29/46)</td>
<td>88% (21/24)</td>
<td>8% (14/176)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>14% (48/339)</td>
<td>29% (48/163)</td>
<td>31% (18/59)</td>
<td>29% (10/34)</td>
<td>27% (10/37)</td>
<td>33% (15/46)</td>
<td>33% (8/24)</td>
<td>0% (0/176)</td>
</tr>
<tr>
<td>Fever</td>
<td>12% (32/339)</td>
<td>26% (42/163)</td>
<td>22% (13/59)</td>
<td>21% (7/34)</td>
<td>30% (11/37)</td>
<td>22% (10/46)</td>
<td>46% (11/24)</td>
<td>0% (0/176)</td>
</tr>
<tr>
<td>Constipation</td>
<td>11% (38/339)</td>
<td>23% (38/163)</td>
<td>24% (14/59)</td>
<td>29% (10/34)</td>
<td>38% (14/37)</td>
<td>20% (9/46)</td>
<td>4% (1/24)</td>
<td>0% (0/176)</td>
</tr>
</tbody>
</table>

Table 2 – This table investigates the question of whether different parasitological infections are associated with different frequency of symptoms reported by patients. All of the lower gastrointestinal parasite-positive patients exhibited a similar distribution of symptoms between diarrhea (~60%), vomiting (~30%), fever (~25%) and constipation (~25%), while parasite-negative and *G. intestinalis* patients exhibited different symptom profiles.
Number of Samples Positive for Specific Enteric Parasites vs. Month of Year in 339 Samples Evaluated in Karachi, 2007

For comparison, US national data from the United States Center for Disease Control is shown above for 12,700 Cryptosporidium spp. cases and 134,000 Giardia intestinalis cases 1998-2002 [19,20]
Geographic Patterns Infections (only in Western nations so far)

Figure 4
Comparison of the prevalence of IBS and chronic abdominal pain to the frequency of detection of Blastocystis in Japan [22,30], Canada [31,32], United States [14,33], Mexico [21,34], and Brazil [22,35].
Patterns Seen at Clinical Labs (US)

Figure 8
The characteristics of common enteric protozoa reported in study of 5792 specimens from US patients collected in 2000 [14]. Studies from the 1980’s reported Blastocystis was usually found as a co-infection with Giardia or Entamoeba histolytica in symptomatic patients [139,140], which was not the case in 2000 [14]. In 2000, the number of symptomatic patients who were found to be singly infected with Blastocystis (400) exceeded the number of samples found positive for Cryptosporidium, Giardia, and Entamoeba histolytica combined. Patients singly infected with Blastocystis were as likely to be symptomatic as patients singly infected with Cryptosporidium (69% vs. 70%) [14].
Goals in IBS-Blastocystis Study

• Identify eradication therapy for long-term cases (Australia, Europe). LTC are highest cost.
• In absence of eradicative therapy:
  – Avoid expensive testing
  – Reduce stress level in patients
  – Avoid expensive medical investigations ($1B on GWS)
• Disease Mechanisms:
  – Reduce symptoms
  – Identify factor that produces spontaneous clearance
  – IL-8, Nitric Oxide, IgA
Confounding Variables in Association Studies

• Many diagnostics tests have low sensitivity (~30% for common clinical diagnostics)
• PCR is improvement, but has limited availability. DNA extraction methodologies and laboratory experience are problematic
• About 50% show symptoms. No single symptom (i.e. diarrhea) associated with infection.
• Multiple other factors can produce identical symptoms.
• What does the “p” value mean at the end?
Figure 1.—Age-specific serum antibody levels to *Giardia lamblia* are shown for 210 persons in the United States (○) and 207 Thai villagers (●). Points represent the mean (± standard error) optical density values by enzyme-linked immunosorbent assay for (A) immunoglobulin M, (B) IgA, and (C) IgG. The number of serum specimens tested in each age group for the US/Thai populations is as follows: cord blood, 13/12; younger than 1 year, 41/41; 1 to 4 years, 80/60; 5 to 19 years, 35/34; 20 to 39 years, 27/28; 40 to 59 years, 21/20; and 60+ years or older, 13/12.
Fig. 1. Scatter plot of the relationship between age and plasma NO$_x$ content in 40 male healthy individuals ($r = -0.877$, $P<0.001$).
Age-Prevalence of Blastocystis spp. Infection in Karachi
Based on 339 Fecal Samples Collected in 2007

Number of giardiasis case reports, by age group and year — United States, 1998–2002

Number of cryptosporidiosis case reports, by age group and year — United States, 1999–2002

* In 1,000s.
† Case reports decreased with increased age.

For comparison, age prevalence data is shown above for 12,700 Cryptosporidium spp. cases (top) and 134,000 Giardia intestinalis (bottom) from a multi-year study of reported US infections published by the United States Center for Disease Control in 2005 [19,20].
What You Can Do With This Model

1. Construct a study to show Blastocystis is strongly associated with IBS using patient selection and appropriate diagnostics.
2. Construct a study to show Blastocystis is not associated with IBS.
3. Make decisions about whether treatment is appropriate in a group, even when a study has found no association.
4. Make informed decisions in reviewing papers.
5. Apply the methodology to other case control studies.
6. Slides focus on Type 1 errors (incorrect rejection of hypothesis).
Examples of Acceptable Levels of CV

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL+</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>70</td>
</tr>
</tbody>
</table>

n=100, 30% exposure, 100% susceptibility, p=4.5E-13

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL+</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>70</td>
</tr>
</tbody>
</table>

n=100, 30% exposure, 50% susceptibility, p=6.12E-10

<table>
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<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>BL-</td>
<td>3</td>
<td>73</td>
</tr>
</tbody>
</table>

n=100, 30% exposure, 50% susceptibility, 75% sensitivity, p=7.79E-07

<table>
<thead>
<tr>
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<th>IBS-</th>
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</thead>
<tbody>
<tr>
<td>BL+</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>BL-</td>
<td>21</td>
<td>55</td>
</tr>
</tbody>
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n=100, 30% exposure, 50% susceptibility, 75% sensitivity, 25% other causes, p=0.002404
### Example of Unacceptable Level of CV

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>90</td>
</tr>
</tbody>
</table>

n=100, 10% exposure, 100% susceptibility, p=5.78E-14

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL+</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>90</td>
</tr>
</tbody>
</table>

n=100, 10% exposure, 30% susceptibility, p=0.00742

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
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</tr>
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<tbody>
<tr>
<td>BL+</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>BL-</td>
<td>1</td>
<td>92</td>
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</table>

n=100, 10% exposure, 30% susceptibility, 75% sensitivity, p=0.012294

<table>
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<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>BL-</td>
<td>24</td>
<td>69</td>
</tr>
</tbody>
</table>

n=100, 10% exposure, 30% susceptibility, 75% sensitivity, 25% other causes, p=0.30. Still have 10% morbidity
## Does Increasing n Help?

<table>
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<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL+</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>900</td>
</tr>
</tbody>
</table>

- **n=1000**, 10% exposure, 100% susceptibility, \( p=1.64 \times 10^{-140} \)

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>BL-</td>
<td>0</td>
<td>900</td>
</tr>
</tbody>
</table>

- **n=1000**, 10% exposure, 30% susceptibility, \( p=1.12 \times 10^{-32} \)

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>20</td>
<td>49</td>
</tr>
<tr>
<td>BL-</td>
<td>10</td>
<td>921</td>
</tr>
</tbody>
</table>

- **n=1000**, 10% exposure, 30% susceptibility, 75% sensitivity, \( p=6.27 \times 10^{-18} \)

<table>
<thead>
<tr>
<th></th>
<th>IBS+</th>
<th>IBS-</th>
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<tbody>
<tr>
<td>BL+</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>BL-</td>
<td>240</td>
<td>691</td>
</tr>
</tbody>
</table>

- **n=1000**, 10% exposure, 30% susceptibility, 75% sensitivity, 25% other causes, \( p=0.000282 \)
Predicted Error in p value vs n
Susc=10%, Sens=75%, Ocauses=25%
Error in p value
vs. Diagnostic Sensitivity

Diagnostic Sensitivity

p value error percent of 0.05

- SUSC=50%, n=100, OC=0%
- SUSC=10%, n=500, OTR=0%
As Promised

• To avoid Type 1 errors:
  – Use larger sample size, higher sensitivity diagnostics, exclude patients with other causes
  – Focus on high susceptibility patients (older age, high IL-8 producers, low IL-10 producers)

• To create Type 1 errors
  – Use lower sensitivity diagnostics
  – Perform testing on patients with other causes (i.e. unsorted healthcare seeking patients, patients in endemic areas, patients during norovirus outbreak)
  – Focus on younger populations
  – Prior examples for Giardia intestinalis / Cryptosporidium spp.: Thai orphanages, US day care, US prisons

• In first example, despite p=0.30, we still had 15% morbidity
Model Predictions

• How to construct a study to show association
  – Use high sensitivity diagnostics, exclude participants with other causes, use susceptible population
  – Most high quality studies after 2006

• How to construct a study to show no association
  – Use low sensitivity diagnostics, include participants with other causes, study children
  – Thai orphanages – *G. intestinalis*, *Cryptosporidium spp.*
  – US day care – *G. intestinalis*
  – US Prisons – *G. intestinalis*

• Model suggests that severe cases can exist in low susceptibility populations (i.e. p=0.20 does not mean mild pathogen or treatment is inappropriate)
Backup Slides
Blastocystis Research Environment

- General agreement at clinical level for treating patients (<3 cases where physician refused treatment 2006-2014)
- *Blastocystis* as a non-pathogen is a minority opinion in literature (16/102 vs. 86/102 as of 2008, most <1994)
- Over 1000 studies to date
- No organizational body with mission to review literature (i.e. no parallel to the IARC 3 degrees). No generally agreed upon mechanism for defining a pathogen (i.e. LD50). So diversity of opinion will remain.


Emerging infectious diseases are not always obvious. Boorom K. Lancet Infect Dis. 2009 Mar;9(3)
