Running title: Koch's postulates for IBD

Title: "Koch's postulates, microbial dysbiosis and inflammatory bowel disease"

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Key words: Koch's postulates, inflammatory bowel disease, Crohn's disease, ulcerative colitis, dysbiosis

Word Count: 3163

Number of figure or table: 01

Over the past 20 years a growing amount of evidence supports the role of microbes and an imbalanced microbiota in inflammatory bowel disease (IBD). While many reviews have been written on the microbiota in IBD, few have considered how they fulfill Koch's postulates. In this review we consider how Koch's postulates might be modified so that they can be fulfilled for polymicrobial diseases and discuss the progress made to date in fulfilling them.

Introduction

Koch's postulates set the standard for proving the role of an organism in a disease. For the postulates to be fulfilled in their current form, the identified organism must (i) be present in all cases of the disease; (ii) be isolated from diseased patients; (iii) cause disease when re-introduced to healthy susceptible animal-model; (iv) then be isolated again from the new host. Polymicrobial diseases or diseases linked to microbial dysbiosis, such as inflammatory bowel disease (IBD), do not fulfill Koch's postulates in their current form. Dysbiosis has been characterized as an alteration in microbial composition or activity that results in an aberrant host response to the microbiota and can be represented by a single or many organisms [1]. Since they were first devised a century ago, Koch's postulates have been modified in order to accommodate new understanding of micro-organisms, such as the inability to culture viruses in the absence of host cells [2]. Therefore, consideration should be given to modify the postulates so as to fit polymicrobial diseases or diseases associated with microbial dysbiosis. As depicted in Figure 1, we propose that Koch's postulates can be fulfilled for IBD with some modifications.

In both clinical and experimental settings there is evidence that support an important role of microbes in both Crohn's disease (CD) and ulcerative colitis (UC), the main forms of IBD. Longstanding examples include the ability of antibiotics to improve

disease symptoms (in some patients); colostomy results in cessation of symptoms in patients; and with few exceptions germfree mice do not develop colitis [3]. Both CD and UC present as an unbalanced immune response to the microbiota, although the nature of the imbalance differs between diseases [4]. While both CD and UC are chronic and relapsing inflammatory conditions of the gastrointestinal track (GIT), they are distinguishable in many ways. For CD, inflammation can occur along the entire GIT with segmental inflammation localized most frequently in the terminal ileum or large intestine. For UC, inflammation is limited to the mucosal layer in large intestine [4]. Crohn's disease can also be further defined based on the anatomical location, which can be associated with different genetic predispositions. These unique distinctions suggest that we should not assume that each disease subtype will conform to Koch's postulates in a similar manner.

The organism(s) fulfilling the postulates

Traditionally Koch's postulates ask for a single organism to be present in all cases of disease. Efforts have been made over the past 100 years to find a causal organism in CD and UC. *Mycobacterium avium paratuberculosis* (MAP) was the first organism to be proposed as causative in CD and continues to be explored [5]. However, several other bacteria have since been identified as potential pathobiont candidates and more recently the role of an overall microbial imbalance without the need for an overt pathogen is supported as a possible disease mechanism [5]. The term pathobiont describes a resident microbe with the potential to be pathogenic but is harmless under normal conditions. It is plausible that more than one mechanism can induce a common outcome of an unbalanced immune response to the intestinal microbiota. Koch's first postulate is that the microorganism must be present in all cases of the

disease. If we were to expand this definition to include the distinct roles of several pathogenic microorganisms individually, as well as an overall dysbiosis, it is quite possible that this postulate can be satisfied. We are unaware of any studies that have taken such an approach to fulfill this postulate, and may well be worthy of future investigation.

Mycobacterium avium* subsp. *paratuberculosis

Mycobacterial avium subsp. *paratuberculosis* was first implicated in CD in 1913 [6]. It is an obligate intracellular organism, and causes Johne's disease in cattle, a chronic granulomatous enteritis that is clinically and pathologically similar to Crohn's enteritis [7]. There have been many studies, which show an association of MAP in CD, but still the exact role of MAP is not clear [8]. A dominant T-cell response to MAP has also been seen in CD patients, and macrophages infected with viable MAP have elevated production of tumor necrosis factor-alpha (TNF-α), a key cytokine released in large amount in CD [9]. A recent perspective has suggested that revisiting the role of MAP in CD is warranted [10]. One argument is that MAP is a very challenging organism to detect and culture, and therefore likely that insufficient efforts have gone to confirm the presence or absence of this organism. Strategies to more efficiently detect MAP will better allow the evaluation of MAP in fulfilling Koch's first postulate. Some common genetic predispositions with Blau syndrome, which has similar granulomatous inflammation as CD and where MAP is also suspect in causing disease [10] provides further support for the role MAP. There is currently a multi-center trial underway to use mycobacterial drugs to treat CD (http://clinicaltrials.gov/ct2/show/NCT01951326?term=rhb104$rank=2). If such efforts are successful, they will certainly solidify MAP in fulfilling Koch's postulates in at least a subset of CD patients.

Adherent-Invasive E. coli

Adherent-invasive *E. coli* (AIEC) is a pathogen that has been isolated from CD patients and particularly abundant in CD patients with ileal involvement [reviewed by Denizot *et al* [11]] . While this strain of *E. coli* can also be found in healthy control subjects, it has several characteristics that would support its role in CD. It has been shown to adhere to and invade human epithelial cells via actin filament and microtubule rearrangement stimulating production of interleukin-8 [12]. Cells cultured with AIEC isolated from CD patients show less trans-epithelial resistance associated with disorganization of F-actin, as well as displacement of both zona occludens 1, and E-cadherin from the apical junctional complex [13]. Furthermore, AIEC have been shown to invade and replicate within macrophages for extended periods [14], supporting the chronic nature of the disease [14]. A study where mice infected with AIEC isolated from a CD patient induced chronic inflammation and intestinal fibrosis characteristic of CD fulfills postulates 2 to 4 [15]. While this study clearly supports the role of AIEC in both inducing and perpetuating the disease, because it is not found in all patients AIEC does not fulfill Koch's 1st postulate in its present form.

Campylobacter

Campylobacter concisus has been identified as an organism of interest in both CD and UC [16]. Analysis of faecal samples using species-specific primers for *C. concisus* have shown that 65% of newly diagnosed children test positive for *C. concisus,* while only 33% of healthy children were positive [17]. Another study showed that DNA from *C. concisus* was detected in more adults with UC (33.3%) compared to healthy controls (10.8%) [18]. Furthermore, there was a positive serum response to *C. concisus* in CD patients, specifically detecting flagellin B, ATP synthase F α subunit

151 [19]. Together these findings showed that *C. concisus* has immunogenic properties
152 and can trigger antibody response in patients with CD [17]. Interestingly, the *C.*
153 *concisus* isolated from CD patients was 500-fold more efficient at invading via the
154 transcellular route than other non-CD isolates [16]. Considering such evidence, *C.*
155 *concisus* might either trigger IBD or exacerbate disease; though further investigation
156 is required. No work to demonstrate UC or CD-like disease symptoms upon re-
157 introduction of this organism into a healthy susceptible model have been published.
158 Therefore, while *C. concisus* seems to have immunogenic properties, the increased
159 abundance of this organism in IBD may simply reflect its ability to survive in an
160 adverse inflammatory milieu [20].

161 ***Dysbiosis without a known pathobiont***

162 In the gastrointestinal tract of healthy individuals there is a mutualistic relationship
163 between the gut microbiota and host [21]. For those with IBD this mutualistic
164 relationship is disrupted and can take a number of forms [22]. Numerous investigators
165 have used advanced cultivation-independent molecular techniques to demonstrate
166 changes in composition of the mucosa-associated and faecal microbiota in IBD
167 patients [23]. One of the challenges has been that many of the changes observed
168 during dysbiosis have been inconsistent between studies [1]. However, some of the
169 more significant commonalities appear to be associated with a decrease in microbial
170 diversity, as well as a specific decrease in *Firmicutes* and an increase in
171 *Enterobacteriaceae* [24-26]. As noted in Figure 1, Koch's 2nd postulate needs to be
172 modified to allow for characterization of the dysbiotic microbial community by
173 molecular methods before it can be fulfilled, as a dysbiotic community cannot be
174 effectively cultured in isolation. One critical aspect of dysbiosis appears to be the
175 inherent loss of anti-inflammatory capacity of the microbiota. For example,

176 *Faecalibacterium prausnitzii,* an organism that is depleted in IBD [27, 28], produces
177 anti-inflammatory metabolites with restorative effects in experimental models of
178 colitis [27]. Contradictory results also exist; including a paediatric cohort study that
179 found increased *F. prausnitzii* in disease [29], which in turn complicate interpretation
180 of the role of this organism. Interestingly, studies in identical twins discordant for
181 disease have revealed that alterations in intestinal microbial profiles are not simply a
182 product of host genotype [30]. It is also important to note that changes in the
183 microbiota of different disease phenotypes (ileal vs. colonic CD) have opposing shifts
184 in the microbiota [30, 31]. While many studies have shown an association with
185 dysbiosis in IBD, prospective studies have yet to demonstrate that dysbiosis precedes
186 disease per se.
187 Whether dysbiosis of some kind precede IBD is still unclear. In IBD there is
188 infiltration of immune cells into the intestinal environment [32]. As such, faecal
189 calprotectin (an indicator of neutrophil infiltration to the intestinal lumen) is used as a
190 biomarker for the diagnosis of IBD [reviewed by Lehmann [33]]. Many of the
191 changes in the microbial community associated with IBD, both with respect to
192 increased pathogen abundance and general dysbiosis, could potentially be explained
193 by prior pro-inflammatory condition. For example, it is well recognized that bacteria
194 within the family *Enterobacteriaceae,* thrive in an inflamed gut [34, 35]. It has been
195 suggested that this is an evolutionary mechanism used by pathogens to outcompete
196 commensal bacteria [35]. Interestingly, the recruitment of an increased number of
197 neutrophils to the gastrointestinal tract in response to *Salmonella* infection results in
198 depletion of Firmicutes that appears to mirror what is observed in IBD [36].
199 Furthermore, it has also been shown that *F. prausnitzii* populations rebound and *E.*
200 *coli* populations fall when inflammation is inhibited with anti-inflammatory

medications [37, 38], which suggests that these hallmarks of IBD dysbiosis are a product of inflammation.

However, in support of Koch's 3rd postulate, it has been shown that a dysbiotic microbiota will trigger and contribute to intestinal inflammation in the absence of an identified pathogen. For example, wild type mice cohoused with Nod2-deficient mice developed more inflammation in chemically induced colitis and show increased expression of apoptotic, necrotic, and tumorigenic genes suggesting transmission of an inflammatory microbiota [39]. Another study by Schaubeck et al. showed that transplantation of microbiotas from diseased mice, but not healthy mice, induced ileitis in susceptible populations of TNF (deltaARE) mice [40]. They further demonstrated this dysbiotic microbiota is responsible for inducing disease in susceptible mice, but not in wild type, suggesting the absence of pathogens. Furthermore, they re-isolated a dysbiotic microbiota from the newly diseased individual supporting Koch's 4th postulate. While this evidence is compelling that a dysbiotic microbiota can induce inflammation, the induction of disease in animal models by the inoculation of dysbiotic microbial communities of patients has not been done.

The susceptible host in IBD

One of the first modifications Koch made to his postulates was in recognition of asymptomatic carriers of disease, thus the inclusion of "susceptible host" to the 3rd postulate. For IBD, this is certainly an important consideration, as genome-wide association studies (GWAS) have revealed over 160 susceptibility human loci; many of them playing critical roles in microbiota sensing/responding pathways, immune regulation and intestinal barrier function [41-44]. There are also variations in genes that correlate with different CD phenotypes. For example, T300A variant in

ATG16L1 shows association with ileal CD, which is linked to disturbances in Paneth cell function [45, 46] and the ability to clear pathobionts [47]. The increasing incidence of IBD would suggest that genotype alone is not sufficient and that changes to the nature of the interaction between microbes and the host is just as critical in the development of intestinal inflammation [48]. Based on the diversity of host susceptibilities, it may be useful to consider each genotype in parallel to the form of microbial dysbiosis. It may well be that different susceptibility loci associate with different microbial triggers and imbalances.

What triggers the disease?

The relatively low concordance rates of UC and moderate concordance rates of CD in monozygotic twins certainly indicate that genetics is only one such predisposing factor rather than an all-encompassing causal explanation [49, 50]. At the same time we also need to incorporate into the model the fact that microbial imbalance is a critical element. We know that most models (from an inflammatory point of view) will not develop colitis under germ-free conditions [5]. In addition, there is also frequent discussion that susceptible genotypes, such as the IL-10 knockout mouse, can lose the spontaneous colitis phenotype when researchers start a model in a new facility (Personal communication, Julia Liu, January 26th 2016). It is also fairly clear that not all bacteria are able to induce disease. One such study by Bloom *et al*. found that selective depletion of some bacteria (in this case *Bacteroides*) can prevent colitis in mice that are well known to spontaneously develop colitis [51]. To support Koch's 3rd postulate they also showed introduction of *Bacteroides* induced disease in the same model, but did not cause disease in a wild type host. Many exogenous factors have been identified that can contribute dysbiosis, but are beyond the scope of this review.

Re-establishment of a healthy microbial community should cure the disease?

When Barry Marshall consumed a vial of *Helicobacter pylori* inducing gastric ulcers he provided evidence for Koch's 3rd postulate [52]. Dr. Marshall then went on to demonstrate that treatment with antibiotics could in fact resolve the self-induced infection. In a similar analogy, if indeed the dysbiotic microbiota (or particular pathogen) are causal in disease, then one might expect that restoration of balance or removal of pathogenic organisms would result in a resolution of disease. While it is true that in some cases of IBD antibiotic treatment can improve outcomes [53], a complete return to health is very rarely experienced without relapse [54]. It is, however, quite plausible that once an imbalance in the immune system is established it cannot be readily restored by rebalancing of the microbiota; as seen in asthma, although a different immune imbalance is involved.

Probiotics and prebiotics

Probiotics and prebiotics have both been used as strategies in an attempt to restore balance to the microbial community [55]. Probiotics are mostly Gram-positive beneficial bacteria that, when ingested, confer benefit to the host through mechanisms such as competitive inhibition of other microbes and increasing mucosal barrier function [56]. The most common probiotics investigated in the context of IBD include *Lactobacillus* spp, *Bifidobacterium* spp, and *Sacchromyces bouladrii*. Indeed, probiotic intervention studies have been shown to extend remission, and reduce disease symptoms [55, 57], but have never been shown to fully restore health or cure the disease.

Prebiotics are non-digestible oligosaccharides that promote the growth of existing beneficial microbes that confer benefit to the host [58]. Some commonly used prebiotics include inulin, fructooligosaccharide (FOS), galactooligosaccharides and

lactulose. In an open labelled trial, use of fructooligosaccharide (FOS), Lindsay et al. showed increased *Bifidobacterium* in faeces associated with an improvement in patients with CD. To date this benefit has not been reproduced in a randomized placebo controlled trial [59].

Antibiotics

In some IBD patients, treatment with antibiotics can reduce the symptoms of disease [5]. Based on the diverse nature of the possible microbial contributors to IBD, it is not surprising that there is inconsistent response to this therapy. The inability to cure disease with antibiotics likely indicates that we have yet to effectively target the right pathogen, or indeed the presence of a single organism is not all that is required for the perpetuation of the disease once initiated. It should also be noted that antibiotics can play a role in causing dysbiosis. For example, *C. difficile* is able to proliferate and cause disease after antibiotic treatment breaks colonization resistance [60].

Faecal Transplantation

Another notable therapeutic approach is faecal microbiota transplantation. This approach has seen great success in the treatment of *C. difficile* associated diarrhea, however only limited studies have been performed in IBD patients. The concept of the therapy is to replace the imbalanced microbiota of an IBD patient with the microbiota of the healthy donor. Borody *et al* showed FMT eradicated CD in refractory IBD in six patients [61], a small trial in UC patients showed effective treatment in some patients [62], and a study with 30 refractory CD patients showed clinical remission in more than 76% after FMT after more than a year [63]. Although further work in this area is needed, these promising preliminary results suggest that re-establishing microbial balance can result in health, therefore contributing to the fulfillment of Koch's postulates.

Conclusion

Over the past decade substantial progress has been made to apply and fulfill Koch's postulates for IBD. It is acknowledged that using the traditional definition of Koch's postulates no single pathogen or form of dysbiosis is likely to be responsible for the disease. However, in the context of IBD, it may be useful to consider a broader modification to Koch's postulates in order to fulfill them. By expanding the postulates it may be possible to allow for multiple forms of microbial triggers, including pathogens or an overall dysbiosis. A second important assumption for IBD is that a common (linear) pattern of microbial dysbiosis or presence of a single pathogen is not necessarily going to satisfy the postulates. However, if we were to expand the list of possible candidate patterns (or even perhaps to accept a non-linear relationship with IBD and the intestinal microbiota) it is likely that Koch's 2nd postulate could be satisfied. It has also been clearly demonstrated that introduction of IBD pathogens or dysbiotic communities can stimulate intestinal inflammation; however, care should be taken in interpreting whether the exposure causes a chronic relapsing disease as opposed to a simple transient inflammatory response. One important issue that has yet to be addressed is whether immune imbalance can be fully restored once triggered. Of course, the ongoing strategy is to demonstrate that promoting a healthy microbiota can be sufficient to prevent the induction of disease. The complexity and multifactorial nature of this disease underscores the fact that current dietary practices, hygiene and frequent antibiotic treatments are all major barriers to the establishment and maintenance of a healthy microbiota.

Acknowledgements: B.P.W. is supported by the Canada Research Chairs Program.

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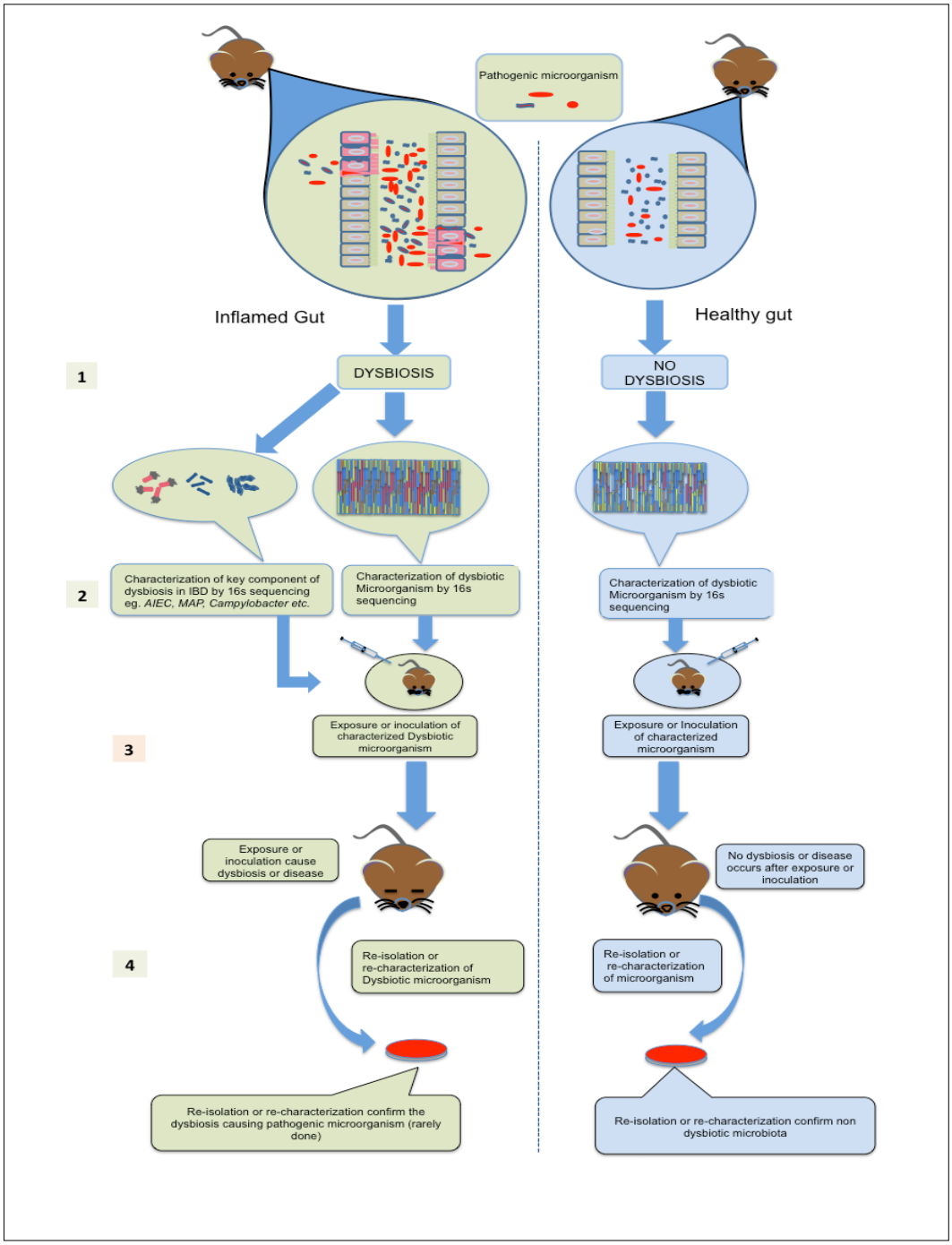
Figure legends

Figure 1. Koch's postulates for microbial dysbiosis and inflammatory bowel disease. We propose that Koch's postulates can be fulfilled with some modifications to the criteria. First is the need to expand the definition from the presence of a single organism in all cases to the presence of several possible pathobionts or dysbiosis in all patients. The modification of Koch's 2nd postulate requires the acceptance of molecular microbial characterization of dysbiosis in place of isolation of the organism. Koch's 3rd postulate also needs to allow for the introduction of several possible single organisms or a dysbiotic community. Finally, while re-isolation of inoculated pathobiont from newly infected patients is possible in some cases, others require the re-characterization of a dysbiotic community by molecular methods.


Pathogenic microorganism
Inflamed Gut
Healthy gut
1
DYSBIOSIS
NO DYSBIOSIS
2
Characterization of key component of dysbiosis in IBD by 16s sequencing eg. AIEC, MAP, Campylobacter etc.
Characterization of dysbiotic Microorganism by 16s sequencing
Characterization of dysbiotic Microorganism by 16s sequencing
3
Exposure or inoculation of characterized Dysbiotic microorganism
Exposure or Inoculation of characterized microorganism
Exposure or inoculation cause dysbiosis or disease
No dysbiosis or disease occurs after exposure or inoculation
4
Re-isolation or re-characterization of Dysbiotic microorganism
Re-isolation or re-characterization of microorganism
Re-isolation or re-characterization confirm the dysbiosis causing pathogenic microorganism (rarely done)
Re-isolation or re-characterization confirm non dysbiotic microbiota

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