Standards for Bacterial Identification by Fluorescence In Situ Hybridization Within Eukaryotic Tissue Using Ribosomal rRNA-Based Probes

To the Editor:


The authors performed fluorescent in situ hybridization (FISH) using a set of 16s RNA-based Cy5 probes and found high concentrations of Bifidobacteria (mean 15 bacteria/1-mm epithelial surface) in normal control subjects and increased intracellular Escherichia coli and Clostridia in inflammatory bowel disease patients. The authors assure us that accurate morphometric measurements were made of each biopsy, that “the total mucosal area was calculated.” Individual bacterial numbers were expressed as numbers of bacteria per millimeter of epithelial surface. Actually, the Cy5 fluorochrome that the authors used is dark red and cannot be perceived with the human eye. Because the only possible way to monitor the fluorescence is with a camera, the microscopy with Axioplan 2 must be performed “blindly,” and morphology can be evaluated only after pictures are taken. However, Cy5 fades quickly, which makes simultaneous searching for optimal location within the biopsy and taking usable pictures difficult. The weak signal of Cy5 makes it impossible to perform orienting shots at low (×400) magnifications to obtain an overview of the biopsy. An inevitable random focusing over the biopsy surface does not allow any morphometric measurements. The following evaluation of microphotographs depends on photoprocessing software. For each setting of photographs, the investigator must choose individually the brightness, contrast, and so forth at which he or she can best evaluate the findings. This adjustment necessarily manipulates the results. The counting of edited signals by 2 observers does not change anything.

In fact, none of the figures presented by the authors shows signals that should be identified as bacteria. Figure 1A shows ≈100 irregular fluorescent clouds that do not have a bacterial morphology and are not DAPI counterstained. The range the authors give for Bifidobacterias is 4 to 56 cells/1-mm biopsy surface. That means that maximally 25 Bifidobacteria can be seen within a single microscopic field at a magnification of ×400. It should be easy for the authors to use arrows to point out what they regard as bacteria in Figure 1A.

The negative control, Figure 1B, shows nothing and therefore cannot be interpreted at all. Normally, each FISH probe, especially Cy5-labeled probes, produces a marked background staining by binding nonspecifically to human tissues. This unspecific background fluorescence makes the human tissue morphology clearly perceivable at fluorescence microscopy and eases the orientation. The phenomenon is general, and the unspecific background fluorescence contrasting human tissues is obvious in Figures 1C through I. In Figures 1A and B, the background fluorescence of the human tissue is absent. The authors do not explain why. Because epithelial morphology cannot be perceived, it is impossible to say from what area Figures 1A and B were taken and how the microphotographs were made. Clearly, they were not made from the biopsy tissue or its surrounding area. All of the other panels claiming to present intracellular bacteria (C, E, G, H, I) demonstrate typical biases of unspecific Cy5 binding to inflammatory cells.

Unlike hybridization signals specific for bacteria, these signals cannot be washed off at any temperature, even at 90°C for 10 min. Despite rigorous washing far above the melting point, the signals preserved their high fluorescence. The demonstrated signals could not be counterstained with DAPI; they hybridized positively with Ec1531 (E coli) and Gam42a (Gamma proteobacteria) but also with Fprau (Fusobacterium prausnitzii), PF2 (Fungi), and Lab158 (Lactobacilli) probes. The fluorescence was much lower when Bac303 (Bacteroides), Erec482 (Eubacterium rectale–Clostridium cocoides), and nonsense probes were used, which may lead to false interpretation when pictures are evaluated separately. (For reasons of length, figures illustrating the latter statements are not shown.)
90°C; they have irregular morphology; they cannot be counterstained with DAPI; and they can be simultaneously hybridized with different unrelated bacterial FISH probes. These biases often are seen within different eukaryotic tissues of healthy people and patients. We observed and documented them in tonsils, vaginal epithelia, nasal epithelia, brain, testes, gallbladder, pancreas, and intestines, as shown in Figure 1. Because most investigators using FISH know and can easily recognize these signals, we never published the data.

Use of new methods such as FISH affords experience in FISH microscopy and adherence to evaluation standards. FISH signals within complex eukaryotic tissues, which generate a vast number of unspecific fluorescence phenomena, must fulfill the following basic criteria for bacterial identification:

1. They must have a morphological form typical for bacteria.
2. They must be detectable in DAPI counterstain, at least in some regions that are not overshadowed by fluorescence of eukaryote cell nuclei.
3. They must hybridize positively with at least 1 other group- or species-specific FISH probe related to the probe of interest.
4. They should not cross-hybridize with FISH probes specific for unrelated bacterial groups and species.

The negative hybridization with a nonsense probe in a separate set of hybridization as performed by the authors gives usable results in pure bacterial communities. When applied to complex eukaryotic tissue, the nonsense probe always generates positive fluorescence signals that are difficult to distinguish from bacteria and can be correctly interpreted only when differently labeled specific bacterial probes are simultaneously used. The signals generated with unrelated probes should not be identical.
when photomicrographs of the same microscopic field are overlaid.

An example of correct evaluation is presented in Figure 2, which demonstrates FISH of a rectal biopsy section from a patient with ulcerative colitis. Two morphologically definite bacterial rods of *Bifidobacteriaceae* (hybridization is performed with Bif164 Cy3) can be seen in Cy3 orange fluorescence (Fig. 2A) and can be assigned within a mucosal bacterial biofilm (Fig. 2B) to universal bacterial signals hybridizing with the Eub338 FITC probe (all bacteria, green signals) and HGC Cy5 probe (Fig. 2C; *Actinobacteria*, red signals). Note that many other Cy5 signals marked with arrows can be seen within mucus and within biopsy tissue in Figure 2C but they have no counterpart either with Bif164 or Eub338 hybridization or in the DAPI counterstain when pictures are overlaid. Therefore, they are obviously of a nonbacterial nature.

FISH of bacteria in eukaryotic tissues that is based exclusively on Cy5 images and uses no DAPI counterstain or simultaneous hybridizations with other related and unrelated FISH probes to verify the fluorescence signals is unacceptable for scientific research.

**Letters to the Editor**

**Alexander Swidsinski, MD**
Department of Gastroenterology
Humboldt University
Charite Campus Mitte
Berlin, Germany

**Mylonaki et al reply:**

We were interested to read Dr Swidsinski’s comments on our article on mucosa-associated flora in the journal (2005;11[5]:481–487). Like Kleessen et al (2002) and, more recently, Kuehbacher et al (2006), we used a Cy fluorochrome-based method for our FISH analysis. We were kindly donated Cy5-labeled probes and were able to maximize the signal-to-noise ratio using a filter block selected from the Zeiss range in a manner similar to that used by Ferri et al (2000). Aware that viewing the samples with fluorescence microscopy is impractical because the signal from Cy5 under these conditions fades rapidly, we used phase contrast microscopy (for surface-associated images) and DIC (differential interference contrast; for lamina proprial images) combined with Axiovision/KS300 software to take and record both tissue coordinates and epithelial/lamina proprial measurements without exposing the sample to Cy5 excitation wavelengths. Once saved to the computer, the fluorescent image was overlaid, using multichannel zvi (Zeiss vision imaging), onto the phase or DIC image, and a precise assessment made as to the location of the signal. There is nothing random or blind about these measurements as coordinates are recorded and used in the multichannel evaluation of the sample. This analysis can be made at magnifications as low as ×200 if a more global assessment is required, but at magnifications of ×400 and above, accurate identification of signal is obvious (Fig. 1).

Dr Swidsinski asserts that none of the figures presented in our article show bacteria. For reasons of space and expense, we were restricted in the numbers of photomicrographs that we could publish, but, as shown in Figure 1C and D, higher-magnification pictures confirm that the signals shown in Figure 1A and in Figure 1 in the May 2005 article have the morphology of bacteria. Our counts were made by 2 experienced observers, one of whom was trained as a microbiologist, and were based on morphological criteria. Counterstaining with DAPI was positive, but was not included in the article because we believed that the images would have added little to readers’ interpretation. To optimize the staining protocol and minimize nonspecific background staining, we conducted a series of preliminary studies involving stringent washes at different ionic strengths and temperatures. Like Dr Swidsinski, and as stated in our article, we confirmed that our bacterial signals hybridized positively with the universal bacterial probe, EUB338. Unfortunately, however, at the time of these studies we had no access to group- or species-specific FISH probes related to the probe of interest. Use of nonsense probes was suggested by one of the original reviewers: When using serial sections and a multichannel zvi, it is possible to subtract hybridization signals to confirm probe specificity and eliminate nonspecific signals. Finally, artificial or false identification of our Cy5 FISH signals as bacteria would not explain the different results we observed for different organisms within the patient groups studied; examples include the increased numbers of epithelium-associated *Bifidobacteria*, *Lactobacillus* and *Bacteroides* compared with other organisms in control subjects (see Table 3 in the original article), and the increase in *Escherichia coli* compared with other bacteria in the lamina propria in patients with inflammatory bowel disease (Table 4 in the original article). Nor would artifact account for the fact that from gentamicin-treated biopsies from patients with ulcerative colitis (but not controls) we have now isolated and serotyped *E coli* (by implication intracellular; unpublished data).

Dr Swidsinski goes further in asserting that Figures 1A and B in the May 2005 article “were not made from the biopsy tissue or its surrounding.” Our intention in Figure 1A in the article, as indicated in its legend, was to illustrate *Bifidobacteria* opposed to the surface of the mucosa; our intention in Figure 1B was to demonstrate, using the nonsense probe for *Bifidobacteria*, the lack of staining for bacteria. All of these pictures were originally submitted to the journal in color, but they were printed in black and white, with a subsequent substantial loss of quality. We agree that in the journal, as opposed to the original images, it is impossible to confirm the source of the images as being mucosa. The color micrographs shown here illustrate these points. Identifying bacteria with arrows was considered unnecessary in our original submission.

We agree with Dr Swidsinski that great care and experience is required...
when undertaking and interpreting studies of this type.

Maria Mylonaki*
Neil Rayment†
David Rampton, DPhil, FRCP*
Barry Hudspith†
Jonathan Brostoff†
*Centre for Gastroenterology
Barts and the London Queen Mary
School of Medicine and Dentistry
London
†Nutritional Sciences Research Division
King’s College
London

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Successful Use of Adalimumab (Humira) for Crohn’s Disease in Pregnancy

To the Editor:

Tumor necrosis factor (TNF) is recognized as an important cytokine in the pathogenesis of Crohn’s disease (CD), and TNF blockade has been effective in the treatment of patients with CD. The first biologic agent shown to induce and maintain remission in patients with CD is infliximab, a chimeric antibody to TNF-α.1 Infliximab is classified as a category B drug for pregnant women. Adalimumab (Humira) is a recombinant human immunoglobulin G1 monoclonal antibody targeting TNF that is approved for the treatment of moderate to severe rheumatoid arthritis as well as psoriatic arthritis. It has demonstrated efficacy for induction of remission in CD.2–4 There are few data about its use during pregnancy. We report a successful pregnancy in a woman with infliximab-resistant CD treated with adalimumab.

A 35-year-old woman with long-standing ileocolonic CD, diagnosed in 1995, had been treated with multiple medications and was either unresponsive to or intolerant of 5-aminosalicylic acid, antibiotics, and purine analogues. She underwent subtotal colectomy and ileorectal anastomosis in 1999. The patient developed recurrent small bowel disease in 2002, and infliximab was started, initially with a good clinical response. However, over time, she required an increase in her dosage and a shortened infusion interval to every 4 to 6 weeks. While on therapy, colonoscopy demonstrated active CD in the distal 45 cm of the neoterminal ileum. Corticosteroids were started. The patient declined treatment with methotrexate and consequently was started on adalimumab 80 mg followed by 40 mg subcutaneously every other week. Her condition improved, and she was able to be tapered off her steroids during the next several weeks. Seven months after starting adalimumab, she became pregnant. After discussions with the patient, a decision was made to continue the adalimumab treatment.
throughout her pregnancy. She had a brief admission at 20 weeks’ gestation for an unexplained fever and abdominal pain treated with a 2-week course of steroids for a presumed flare of her CD, without any endoscopic studies or imaging performed. She delivered a normal, full-term male infant vaginally. The patient remains on adalimumab and is breastfeeding her newborn. The baby is now 6 months old with normal growth and development.

The treatment of inflammatory bowel disease in pregnancy is a concern for both patients and their physicians. It is accepted that maintenance of remission of inflammatory bowel disease is associated with a successful pregnancy and that active inflammatory bowel disease has been shown to be a factor associated with poor pregnancy outcomes. Accumulating data support the safety of intentional or inadvertent use of infliximab in pregnancy. These data suggest that the benefits of infliximab in achieving response and maintaining remission in mothers with CD may outweigh the potential risk to the fetus from exposure to the drug.

Adalimumab is a category B drug in pregnancy. Small animal studies in pregnant monkeys have not demonstrated adverse effects of adalimumab. There is 1 English language case report describing the successful use of adalimumab started before conception and continued throughout the pregnancy. In this report, the patient also suffered from infliximab-resistant CD, and despite severely active disease at conception and moderate activity in the third trimester, the pregnancy was uncomplicated.

When evaluating medications in women who are pregnant or contemplating pregnancy, it is important to address the safety regarding the ability to conceive, teratogenicity, risk of miscarriage, and complications for the child. This decision should be made on an individual basis. In this case, adalimumab induced and maintained remission of infliximab-refractory CD without any apparent side effects to the mother or child. A registry of women who received adalimumab during pregnancy has been initiated, and physicians are encouraged to report such patients (toll-free 877-311-8972).

Daniel S. Mishkin, MD, CM*  
William Van Deinse, MD†  
James M. Becker, MD‡  
Francis A. Farraye, MD, MSc*  
*Section of Gastroenterology  
†Prime Care Biddeford, Maine  
‡Department of Surgery Boston Medical Center Boston, Massachusetts

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Cardiac Involvement in Children with IBD During Infliximab Therapy

To the Editor:

Infliximab (IFX), a chimeric monoclonal antibody against tumor necrosis factor-alpha, has been shown to be effective for the treatment of severe inflammatory bowel disease (IBD) in adults as well as in children. Patients who receive IFX may exhibit unwanted adverse effects such as acute and delayed infusion reactions, infections, and serological and clinical autoimmune events. In a large cohort study in 500 consecutive adults treated at the Mayo Clinic, IFX-related infections were found in 8%, including 20 patients with serious infections, whereas infusion reactions occurred in 1.5% of 594 infusions administered to 111 pediatric patients.

In 2003, Kwon et al reported 38 adult patients who developed new-onset heart failure and 9 who exacerbated preexisting heart disease during IFX therapy. Recently, others have reported new-onset heart failure in patients undergoing IFX therapy without previous history of cardiovascular disorders: all of the patients improved after discontinuation of infusions. A sudden death was reported to have occurred 18 h after a single infusion in a 64-year-old man without heart failure; at the autopsy, organic causes of death such as myocardial damage, pulmonary or cerebral edema, rupture of aneurysms, pulmonary embolism, and internal bleeding were excluded. All reported patients developing new-onset heart failure upon IFX exhibited normal cardiac parameters before biological therapy. Evaluation of cardiac function has never been described in children undergoing IFX therapy.

We wish to report the preliminary results of a study aimed at evaluating heart function through echocardiography and electrocardiography monitoring in children with IBD during IFX therapy. This study was carried out on 26 subjects. Twelve had IBD (4 females, median age 14.0) with Crohn’s disease and 3 with ulcerative colitis; 11 received IFX therapy; 14 age- and sex-matched healthy subjects served as controls.

Doppler echocardiography was carried out to assess ejection fraction, left ventricular diastolic diameter, left ventricular systolic diameter, thickness
of interventricular septum, and posterior wall and the respective Z-scores. Computed standard 12-lead electrocardiogram was performed to calculate QT, QT correct interval length, and QT dispersion as bioelectrical risk factors of arrhythmias and sudden death; 10-min EGG monitoring in 2 leads was performed to evaluate sympathovagal balance by time and frequency domain indexes of heart rate variability.

Heart involvement was present in 7 of 12 children with IBD: 2 showed a dilative echocardiographic pattern and 5 a septal hypertrophic echocardiographic pattern (1 of them with a bicuspid aortic valve disease). All of these patients received IFX therapy and 5 also received long-term administration of corticosteroids. No differences were found in heart rate variability indexes as compared with controls, whereas a positive correlation between QT dispersion and LVDD and LVSD was found, thus suggesting an increased risk to develop cardiac arrhythmias. Because of lack of cardiovascular screening, we could not exclude the presence of asymptomatic myocardial disease before IFX therapy.

Our preliminary results suggest that assessment of heart function and structure should be included in the monitoring of children undergoing IFX through prospective studies that must recruit a large patient population. The latter should be stratified in subgroups according to different clinical phenotypes and therapeutic strategies. Currently, electrocardiography and imaging studies, such as ultrasound and magnetic resonance, appear to be the most suitable tools to detect potential bioelectrical instability and structural damage of the heart during IFX therapy.

Maria Barbato, MD
Mario Curione, MD
Franca Viola, MD
Paolo Versacci, MD
Fabiana Parisi, MD
Silvia Amato, MD
Salvatore Cucchiara, PhD
La Sapienza University
Rome, Italy

REFERENCES