

Culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from the Blood of Patients with Crohn's disease: A Follow-Up Blind Multi Center Investigation

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Abstract: Blood samples from 58 subjects were blindly coded and investigated by three independent laboratories for the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) using MGITpara specialized media and nested PCR. Consequently, viable MAP was detected in 22/40 (55%) IBD (11/20 CD and 11/20 UC) compared to 4/18 (22%) NIBD ($P < 0.009$). At least two centers detected MAP in 41% IBD samples compared to none (0%) in NIBD ($P < 0.0001$). No sample was positive by all three centers. Despite result variability between centers, the study strongly demonstrates that using MGITpara culture media and nested PCR are essential for successful detection of MAP in human blood. Overall, the study supports a mycobacterial role in CD pathogenesis.

INTRODUCTION

Earlier in our lab, we reported the culture and detection of viable *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from the blood of 43% Inflammatory bowel disease (IBD) patients (14/28 Crohn's disease patients (CD) and 2/9 Ulcerative colitis (UC) patients) compared to none (0%) in 11 non-IBD (NIBD) patients [1]. In the same study, we identified MAP DNA in uncultured blood samples from 45% IBD compared to 22% NIBD. These results were complimentary to our previous reports of detection of MAP from tissue and breast milk from CD [2,3]. However, the detection of MAP in the blood of CD patients is intriguing and resulted in vigorous debate in the literature. The authors were challenged to reproduce the blood study in a blind multi center investigation. Consequently, teams of Mycobacteriologists from the University of Central Florida (UCF, Orlando Florida, USA), University of Wisconsin (UWS, Madison, Wisconsin, USA) and the Center for Disease Control and Prevention (CDC, Atlanta, Georgia, USA) have agreed to receive Coded EDTA-blood samples from the University of Florida (UF, Gainesville, Florida, USA) and to follow the culture and nested PCR protocol published in the original Study [1].

MATERIALS AND METHODOLOGY

UF recruited the consented matched age and gender subjects following IRB regulations, collected the blood samples, disseminated the samples to all centers at the same

time and under the same conditions and secured the sample identity until results from all centers were reported. Over all, four 6-mL EDTA peripheral blood tubes were collected from each of 58 subjects participated in the study. The testing in each center was performed with minimum variability using MGITpara specialized culture media. One blood tube sample from each subject was also sent out to a local reference diagnostic laboratory for routine blood culture. Following six months of MGITpara culture incubation, culture aliquots were subjected to DNA extraction and nested PCR analysis as described earlier [1]. The results were then collected and tabulated by the Clinical Coordinator at UF.

RESULTS

At the conclusion of the study, the codes were identified and the results were shared among all investigators. The results are as follows: 1) Routine blood culture by the reference diagnostic laboratory reported no microbial growth at all in the blood samples from all 58 subjects; 2) MGITpara blood culture testing performed by UCF, UWS and CDC detected viable MAP in 22/40 (55%) IBD (11/20 CD and 11/20 UC) compared to 4/18 (22%) NIBD ($P < 0.009$); 3) specifically, UCF detected MAP in 13 (33%) IBD (4 CD and 9 UC) compared to 3 (17%) NIBD, UWS detected MAP in 15 (38%) IBD (6 CD and 9 UC) compared to 1 (6%) NIBD and CDC detected MAP in 5 (13%) IBD (4 CD, 1 UC) compared to none (0%) from NIBD; 4) At least two centers detected MAP in 41% IBD samples compared to none (0%) in NIBD ($P < 0.0001$). No sample was positive by all three centers.

DISCUSSION

The results clearly demonstrate that MAP bacteremia can be cultured and detected by three independent laboratories

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with expertise in mycobacterial culture and at least by two labs with MAP specifically. Most recently, Kirkwood *et al* [4] also reported the detection of MAP in the blood from children with early-onset CD. The MAP detection rate in IBD patients reported from this blind multi center investigation confirms our earlier published report. The absence of microbial growth in all samples using routine blood culture is not surprising. Blood is sterile and routine blood culture media usually detect most bacteremia except fastidious pathogens like MAP which requires special nutritional supplements and long term incubation. Result variability between different labs is not unique and can be caused by several factors. First, blood sampling error is considered a serious cause of result variability and this may explain why any blood culture test is always done in duplicate and one positive is considered satisfactory. Second, we used only 6.0 mL of blood sample per lab compared to the 20.0 mL duplicate-bottles usually used in standard routine blood culture testing. Third, variation between facilities and technicians' skill could play a role in result variability especially that MAP cells in circulation is cell wall deficient fragile form and found in limited numbers. Despite these challenges, the study shows, even with limited success, that MAP is present and can be detected in blood samples from same patients at least by two independent laboratories. The study strongly demonstrates that using MGITpara culture media and nested PCR are essential for successful detection of MAP from human blood. Other studies that reported a lack of detection of MAP DNA in human blood may suffer from poor methodology design especially by using inappropriate culture media or by relying only on regular PCR techniques. Since our original report of culturing MAP from the blood of CD patients was

challenged for reproducibility, the data here clearly demonstrate that MAP can be detected in human blood by independent laboratories only when MGITpara and nested PCR are used. The burden now is on other methodologies which failed to detect MAP in human blood. Such methods may be appropriate for detection of MAP from animal samples but should be declared to be incompetent for culture of human MAPbacteremia. Moreover, if new methods are employed for investigating the presence of MAP in human blood, they should be used in parallel to the methods described in this study. Only then, the presence or absence of MAP in human with IBD can be accurately reported. Overall, the study clearly supports a MAP role in CD pathogenesis.

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