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# Extracellular Components in Culture Media of *Mycobacterium Avium* Subspecies and *Staphylococci* with Implications for Clinical Microbiology and Blood Culture

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**Abstract** Rapid culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from patients remain a challenge. During the process of developing a rapid culture method for MAP, we found extracellular bacterial DNA, RNA and proteins for MAP, MAH (*Mycobacterium avium* subspecies hominissuis) and other bacteria such as *Staphylococcus aureus*, and *Staphylococcus pseudintermedius* in the supernatants of our liquid culture media. When cultured on the solid media plate, there are a limited number of colonies developed for MAP and MAH disproportionate to the expected numbers of mycobacterial growth. The presence of extracellular components within the liquid culture media raises the possibility of an unknown biological process or a cell death process during the culture that has not been previously described. Based on the presence of extracellular bacterial DNA, RNA and proteins in the liquid media of various bacterial/mycobacterial cultures, and the fact that automated clinical blood culture system consistently gives rise to a low yield, we tested 62 blood culture specimens that have been reported negative by a routine automated blood culture method after 5 days of incubation. We used high sucrose culture media and molecular diagnostic techniques to test these negative culture bottles, and we found a large percentage of bacterial growth by high sucrose culture media (32%) and by molecular PCR amplification using 16S rDNA primers and DNA sequencing (69%). The bacteria identified by PCR/sequencing methods from these culture-negative bottles are diverse and may play roles in pathogenesis of these clinically ill patients. The sensitivity of detection by an alternative culture media and the molecular PCR/sequencing method are significantly higher than by routine automated blood culture. We suspect the low yield of automated blood culture in hospital setting may result from cell death of bacteria under the culture conditions. Our study demonstrates a potential alternative bacterial growth/death pattern in liquid media and provides an area of needs for more sensitive and faster diagnostic tools to guide clinical practice and improve the outcome of sepsis management.

***Keywords:*** *Mycobacterium, Paratuberculosis, Hominissus, Programed cell death, Automated blood culture, Blood culture sensitivity, PCR, 16S rDNA sequencing*

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## 1. Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is known to be a slow grower with a special requirement for mycobactin as a growth factor in the culture media [1,2]. MAP has been extensively studied in Johne's disease, a veterinary condition in cattle and sheep, and it has been associated with Crohn's disease (CD), although the association is controversial [2]. The technical difficulty of growing MAP in culture lead us to search for an alternative method to grow MAP more rapidly to determine the relationship of MAP and CD and to multiple other–conditions such as multiple sclerosis, and type I diabetes mellitus [3,4,5]. MAP growth in solid media or liquid media requires an extended period of time (up to 1 years). This slow growth pattern of MAP suggests there may be a specific cell death process during MAP culture. The best known mechanism of a cell death is the transiting of bacteriophage from pro-phage to lytic cycle of mature phage during growth of *E. coli* [6]. The phenomenon of the lytic cycle of *E. coli* growth with bacteriophage has been described as a specific type of programmed cell death (phenoptosis) [7]. MAP phages (Mycobacterium phage vB_MapS_FF47, Genbank # NC_021063) have been previously identified and isolated without known implication or functions [8]. Another

mycobacteriophage with highly homologous DNA sequence to phage FF47, phage Muddy, had also been isolated from soil samples from Africa (Genbank # NC_022054). There are other mycobacteriophages known to infect MAP as well as other Mycobacterial species [9]. There are numerous pro-phage sequences within the genomes of many different bacteria/mycobacteria including MAP/MAH based on the DNA sequence analysis, and whether these pro-phage sequences will biologically transit to mature phages remain largely unknown [10,11].

MAH is one of the closest members within the subfamily of *Mycobacterium avium* subspecies with significant homology in structure and functionalities to MAP. However, MAH is a much faster grower with significant clinical implications in immune compromised patients such as cancer patients undergoing chemotherapy and HIV/AIDS patients [12,13]. We have previously isolated a strain of MAH, MAH-*ashley*, from a Crohn's patient [14]. MAH-*ashley* grows fast with colonies in 48-72 hours on the solid media plate. We used *Staphylococcus aureus* and *Staphylococcus pseudintermedius* as controls to show similar growth are present in other bacteria.

In the current study, we accidentally discovered the presence of free bacterial DNA, RNA and proteins within the supernatants of the liquid media, and we suspect the presence of the cellular components such as DNA, RNA and proteins in the culture supernatants may represent a specific cell death process, although we do not fully know. We further tested clinically automated blood culture-negative bottles for the presence of bacterial DNA by using PCR and sequencing techniques with potential implication of clinical microbiology and bacterial culture.

# 2. Materials and Methods

## 2.1. Bacterial Culture in Liquid and Solid Media

MAP *Dominic* strain (ATCC Cat: 43545) was purchased from ATCC and maintained in the modified liquid and solid media plate described previously [14]. MAH was isolated from the blood of a Crohn's patient by our laboratory previously and was maintained in liquid and solid media as previously described [14]. MAH isolated from our laboratory, MAH-*ashley*, has been further characterized by partial genomic sequencing for IS1245 and full genomic sequencing (Next generation sequencing, Illumina Miseq Sequencing System) from West Virginia University (WVU) Genomics Core facility, Morgantown, WV, and the sequencing data were analyzed and compared with the existing MAH and other Mycobacterial sequences in Genbank (unpublished data). The DNA sequence of MAH-*Ashley* is highly (99-100%) homologous to Mycobacterium avium strain 104 and MAH-TH135 by partial genomic sequencing using primers for 16S rDNA and IS1245 [14], and both were isolated from HIV patients [12,15]. MAH-*ashley* is a fast grower with visible colonies on the solid media plate within 48-72 hours. Three different types of media, liquid medium, regular solid medium plate, and high sucrose medium were routinely used for culture of MAP and MAH [14]. *Staphylococcus aureus* was isolated from the blood of a chronically ill patient through high sucrose medium culture method based on the Middlebrook 7H10 media, and this isolate can grow in all culture media including Brain-heart infusion broth/plate, Tryptic Soy broth/plates as well as chocolate agar plate. Partial genomic DNA sequencing using 16S rDNA primers and subsequent full genomic sequencing by NGS at WVU Genomics Core Facility were also performed for characterization of the isolate (unpublished data). *Staphylococcus pseudintermedius* was isolated from the skin wound of a domestic dog, and the growth behavior of this isolate is similar to that of *S. aureus*. Similar partial genomic sequencing and full genomic sequencing by NGS were also performed on this isolate of *S. pseudintermedius* for confirmation (unpublished data). All specimens sent to NGS sequencing by WVU Genomics Core Facility was extracted from the culture media supernatant for the purpose of identification of bacteriophage DNA sequences. All culture media components were purchased from BD Biosciences (Middlebrooke 7H9, 7H10, Tryptic Soy media, Brain-heart infusion media, media additives), and prepared in our lab according to the manufacturer's instruction. Mycobactin J was obtained from Allied Monitor Inc., MO.

## 2.2. DNA, RNA and Protein Extraction and Gel Electrophoresis.

All DNA, RNA and protein isolation was carried out as described previously using standard methods except for pre-treatment of the bacterial/mycobacterial cultures with acetone [6,14]. DNA and RNA were visualized on the 0.7% agarose gel electrophoresis for integrity and visual estimation. All proteins were visualized on 10% SDS-polyacrylamide gel electrophoresis.

## 2.3. PCR Analysis of Bacterial Culture and Mycobacterial CULTURE

PCR amplification of specific genes for MAP, MAH and 16S rDNA was performed as described previously [14]. Partial genomic sequencing of PCR amplicons was performed at Eurofins Genomics facility in Louisville, KY. The partial DNA sequences generated from the PCR amplicon sequencing were compared against Genbank using the existing BLAST nucleotide search engine at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 2.4. Routine Automated Blood Culture and Subsequent Analyses

Routine automated blood culture bottles from bioMerieux BacT/Alert 3D clinical system were obtained from a local hospital after 5 days incubation, and these blood culture bottles were reported as "negative" and to be discarded. We only tested aerobic culture bottles as a proof of principle, and no anaerobic or fungal cultures were examined. An IRB expedited review approval was obtained. The culture bottles were opened in the level 2 biosafety cabinet hood, and two 200 ul aliquots were removed: one aliquot was used for high sucrose medium culture and another aliquot was for DNA/PCR analysis. Cultures on the high sucrose media slope were kept for 72 hours, and the results were

collected/recorded. A longer incubation time was not tested. The high sucrose culture medium was developed in our laboratory as described previously with the base media Middlebrook 7H10 agar supplemented with 0.1% yeast extract, 3% glycerol and 20% sucrose for induction of cell wall deficient bacteria/mycobacteria [14]. The culture media were prepared in our laboratory and autoclaved for 121$^0$C for 20 minutes with pressure of 21 psi (standard liquid cycle). An aliquot of 200 ul was taken from the culture bottle and directly inoculated on the slope of high sucrose culture media. The inoculated blood culture on the slopes were incubated in a 37$^0$C incubator for 72 hours, and examined by direct visualization of colonies on the slopes. Visible colonies on the slopes are reported as positive.

An additional 200 ul blood culture was taken from the blood culture bottle, and transferred to a sterile Eppendorf micro-centrifuge tube. The blood culture is centrifuged at 12000g for 5 minutes to separate the cell pellet/debris from the supernatant. An aliquot of 20 ul of the supernatant was directly loaded on 0.8% agarose gel for electrophoresis. Genomic DNA from the cell pellets was extracted by using the Quick & Easy Genomic DNA Kit from PZM Diagnostics, LLC (Charleston, WV) according the manufacturer's instruction. Briefly, the cell pellet was resuspended with Solution I, and washed once with Solution II. The final cell pellet was resuspended in Solution III before being heated at 95$^0$C for 15 minutes. The aliquot is centrifuged at 12000 g for 2 minutes, and 0.5 ul of the supernatant is directly used for PCR amplification in 25 ul total volume. PCR primers were based on the 16S rDNA primer set described previously (forward primer: 5'- GAG GAA GGT GGG GAT GAC G-3', reverse primer : 5'- AGG CCC GGG AAT GTA TTC AC – 3') [14]. PCR amplification was visualized on 6% non-denaturing polyacrylamide gel or 1.5% agarose gel electrophoresis, and the PCR amplicons were stained with ethidium bromide [6]. Direct digital photographs were taken. PCR amplicons were sent to Eurofins Genomics services, Louisville, KY for direct sequencing and the DNA sequences were subjected to BLAST search of Genbank at NCBI.

# 3. Results

## 3.1. Presence of DNA, RNA and Proteins of MAP, MAH, *S. aureus* and *S. pseudintermedius* in Liquid Culture Media (Supernatant)

We maintained the culture in liquid media for MAP, MAH-*ashley*, *S. aureus* and *S. pseudiointermedius* in their respective culture media suitable for growth. MAP and MAH-*ashley* were maintained for 3-weeks and 3 days respectively in Middlebrook 7H9 broth with various supplements, and *S. aureus* and *S. pseudintermedius* were cultured in the brain-heart infusion (BHI) broth for 48 hours. An aliquot of 20 ul of culture medium after centrifugation was loaded on 0.7% agarose gel for electrophoresis with ethidium bromide (Figure 1A). There are clear DNA bands over 10 kb in size in all the culture tubes, indicating the presence of specific DNA fragments in the culture media supernatants. Culture medium from *Escherichia coli* (ATCC 25922) or no bacterial inoculates (negative control) showed no evidence of DNA or any band (not shown).

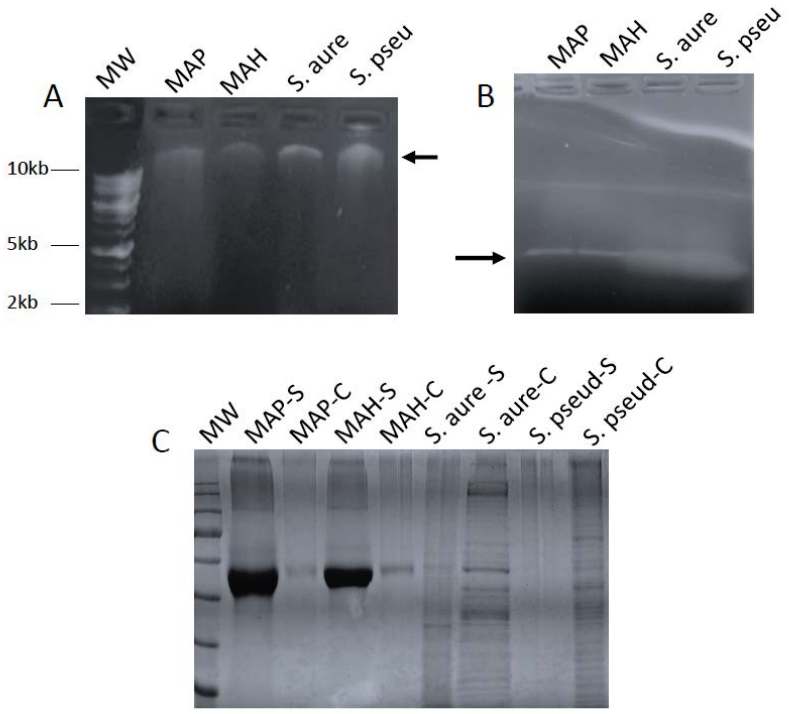


**Figure 1.** The presence of DNA, RNA and proteins in the culture media of MAP, MAH, S. aureus, and S. pseudintermedius. A: The culture media supernatants (20ul) were directly loaded on 0.7% agarose gel with ethidium bromide after centrifugation. MW is 1kb molecular weight marker with maximal 10 kb. B: The culture media supernatants (40 ul) were directly loaded on 1.2% agarose formaldehyde gel with ethidium bromide. C: The culture media supernatants and cell pellet lysates (20 ul) were loaded on 10% SDS-PAGE. The high intensity band in MAP-S and MAH-S represents added albumin of the OADC supplement in the 7H9 culture media. Media control shows no DAN, RNA or proteins (not shown)

Similarly 20 ul aliquots of culture media supernatants were loaded on 1.2% agarose formaldehyde gel using MOP buffer (Figure 1B), and there are specific RNA within all the culture media. Aliquots of 20ul culture media supernatants were loaded on 4%/10% stacking SDS-polyacrylamide gel for protein analysis and stained with Coomassie blue (Figure 1C). There are clearly multiple bacterial proteins within the supernatants of all cultures after the centrifugation, in addition to DNA and RNA (Figure 1A and 1B). There were more proteins in the supernatants of MAP and MAH cultures than the cell pellet lysate (Figure 1 C). The control media without bacterial inoculation do not show any DNA, RNA or proteins on the gel electrophoresis (not shown). DNA was extracted from the culture media supernatants and sent to NGS sequencing and the data generated from the Illumina Midseq sequencing revealed only the host bacterial DNA (WVU Genomic Core Facility). There is no evidence of bacteriophage DNA sequences (Data not shown).

## 3.2. Presence of Bacterial DNA in Clinical Automated Blood Culture

Based on the results described above, we examined 62 blood culture bottles obtained from the local hospital using the bioMerieux BacT/Alert 3D automated blood culture system. We only examined the aerobic culture bottles for proof of principle. These blood culture bottles were to be discarded after 5-day incubation and the "negative" culture results had been reported clinically. Two separate aliquots of liquid media from the automated culture bottles were obtained: one aliquot was inoculated on the slope of high sucrose media as described previously, and one aliquot was used for DNA and PCR/sequencing analysis. The slope culture was kept for 72 hours at 37°C and the results of visible colonies were recorded. We only report the presence (positive) or absence (negative) of visible colonies on the media slopes, and we did not count the number of colonies to quantify the bacterial growth. The patients' clinical and demographic information was not obtained. One aliquot of the culture media was centrifuged and the supernatants were directly used for 0.7% agarose gel electrophoresis (Figure 2 A). There are multiple clinical samples without the presence of DNA bands (Figure 2 A). It is difficult to determine if the DNA on the gel represents the host human DNA or the bacterial DNA or the mixture of both. The cell pellets were used for DNA extraction and PCR analysis using the 16S rDNA primer set as described [14] (Figure 2B). There are multiple samples with positive PCR amplifications, and many negative PCR reactions (Figure 2 B). The positive PCR amplicons were sent for sequencing analysis to Eurofins Genomic services. The BLAST results of the PCR amplicons are listed in Table 1 for the best match identification. As in Table 1, there were 20 positive culture results with visible colonies on the high sucrose media containing 20% sucrose (20/62, 32.2%), and 43 of the 62 culture bottles were positive by PCR using the 16S rDNA primer set as described (43/62, 69%). The bacteria species identified by DNA sequencing of the PCR amplicons were diverse and listed as in the last column in Table 1.

**Table 1. Culture on high sucrose media slants and PCR/sequencing identification of blood culture negative bottles**

| Patient | High sucrose | PCR | Sequencing ID |
|---|---|---|---|
| 1 | - | + | Enterococcus sp. |
| 2 | + | + | Propionivibrio sp |
| 3 | - | + | Streptococcus sp. |
| 4 | - | + | Streptococcus oralis |
| 5 | - | + | Clostridium sp. |
| 6 | - | - | |
| 7 | + | + | Streptococcus sp |
| 8 | - | + | Streptococcus alactolyticus |
| 9 | - | - | |
| 10 | - | + | Streptococcus suis |
| 11 | - | + | Streptococcus suis |
| 12 | - | + | Streptococcus sp. |
| 13 | - | + | Streptococcus suis |
| 14 | - | + | Desulfobulbus sp |
| 15 | - | + | Streptococcus suis |
| 16 | - | + | Streptococcus sp |
| 17 | - | + | Streptococcus pneumoniae |
| 18 | - | - | |
| 19 | + | - | |
| 20 | + | + | Streptococcus sp |
| 21 | + | + | Streptococcus gallinaceus |
| 22 | + | + | Streptococcus constellatus |
| 23 | - | + | Streptococcus peroris |
| 24 | + | + | Propionivibrio sp. |
| 25 | - | + | Uncultured bacterium clone |
| 26 | + | + | Streptococcus suis |
| 27 | - | + | Uncultured bacterium clone |
| 28 | - | + | Lactobacillus bifermentans |
| 29 | - | - | |
| 30 | - | + | Streptococcus mitis |
| 31 | + | - | |
| 32 | + | + | Streptococcus sp. |
| 33 | - | + | Streptococcus panodentis |
| 34 | - | - | |
| 35 | - | - | |
| 36 | + | + | Propionivibrio sp |
| 37 | + | + | Streptococcus sanguinis |
| 38 | - | + | Actinobacterium clone |
| 39 | - | - | |
| 40 | + | + | Brevibacillus sp. |
| 41 | - | + | Uncultured spirochete clone |
| 42 | - | - | |
| 43 | - | + | Streptococcus sp. DP42 |
| 44 | - | - | |
| 45 | - | - | |
| 46 | + | - | |
| 47 | + | + | Streptococcus mitis |
| 48 | - | + | Lactobacillus aviarius |
| 49 | + | - | |
| 50 | + | + | Paenibacillus sp |
| 51 | - | + | Lactococcus sp. |
| 52 | + | - | |
| 53 | - | + | Haemophilus sp. oral clone |
| 54 | - | - | |
| 55 | - | + | Streptococcus sp |
| 56 | - | + | Flavobacteriales |
| 57 | - | + | Enterobacter cloacae |
| 58 | - | + | Enterococcus pseudoavium |
| 59 | - | - | |
| 60 | - | - | |
| 61 | - | - | |
| 62 | + | + | Proteus mirabilis |
| Total 62 | 20+ | 43+ | |

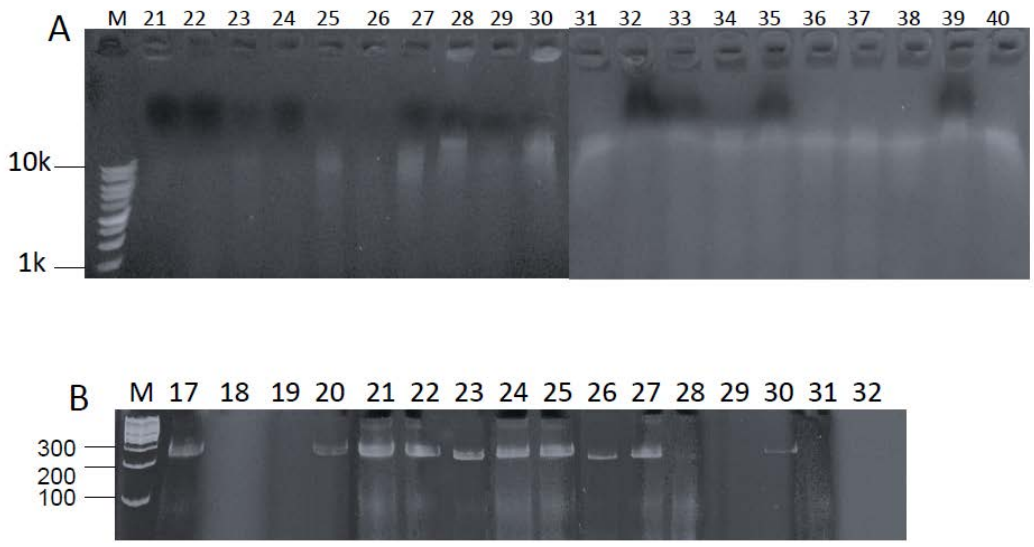


**Figure 2.** The presence of bacterial DNA within the culture media and PCR amplification from the automated blood culture system. A: Automated blood culture media (20 ul) directly loaded on 0.7% agarose gel electrophoresis after centrifugation. MW is the 1kb molecular weight marker. B: 6% non-denaturing acrylamide gel electrophoresis of PCR amplification using the DNA from the automated blood culture bottles. MW is the 100 bps molecular weight ladder.

## 4. Discussion

MAP growth is an area of great interest in the diagnosis of CD in humans and Johne's disease in cattle and sheep. The technical difficulties are multiple, and the established method for MAP culture is clinically impractical due to the prolonged period of incubation. This technical challenge makes it difficult to convincingly demonstrate the role of MAP in the pathogenesis of CD. Assuming that the doubling time of MAP replication is 14-20 hours [1], a single cell may be estimated to yield a visible colony in less than 30 days. To incubate the culture for 6 months is difficult to imagine from the standpoint of basic cell division. This suggests that there is an alternative unknown growth behavior of MAP. The DNA, RNA and proteins present in the liquid media (extracellular) suggest a lytic growth pattern with or without a phage. We also noted the same growth patterns in MAH, *S. aureus* and *S. pseudintermedius*. We have identified a strain of *E. coli* from a patient with nephrotic syndrome with similar growth behavior. We also filtered the culture supernatant with 0.2 micron sterile syringe filter, and we didn't see colonies on the plate after filtration (data not shown). The presence of bacterial extracellular DNA, RNA and proteins in the culture media is a phenomenon unknown to us, and we are not aware of any previous report of free extracellular contents (DNA, RNA and proteins) present in the media supernatant. These features seem to fit the description of "phenoptosis". Phenoptosis is primarily described in mammalian cells with a critical role of mitochondria, but the bacteria in general lack mitochondria. Alternatively, there could be a specific bacterial programed cell death pathway triggered in the culture condition, leading to bacterial lysis [16]. Exactly what the biological process these free extracellular components in the culture media represent is unknown to us, and to our knowledge, there is no documented literature of this peculiar process in clinical culture setting. Protein-leaky mutants of *E.coli* have been previously described with chemical mutagenesis, and these leaky mutants were reported to form blebs on the cell surface without lysis of the bacteria [17,18]. But these protein-leaky mutants of *E.coli* reduced approximately 15% of the protein contents. No bacterial DNA or RNA leakage was reported or studied.

We investigated whether extracellular components are present in the patients with routine automated blood culture. Clinically it is well known that the yield of the automated blood culture system ranges from 5 to 15% in hospital patients. This low yield could be due in part to the bacterial growth behavior. We used a high sucrose media with high osmolarity (20% sucrose), and this high osmolarity media increased the yield of culture significantly. High osmolarity media induce the growth of cell-wall deficient bacteria/Mycobacterial growth [19]. The presence of free DNA on gel electrophoresis from the media of the culture bottles is difficult to discern since there is human host DNA from the blood cells and/or cell-free circulating DNA, and the question of the DNA bands/smear on the gel representing host human DNA or bacterial DNA or a mixture of both requires either PCR analysis of specific targets or metagenomic DNA sequencing. It is difficult to compare the results of the gel electrophoresis of the culture media to those of PCR amplification, since PCR amplification using 16S rDNA primers is specific to bacterial DNA. The presence of bacterial DNA by PCR/sequencing in clinically culture negative patients raises the question of whether automated routine blood culture systems have sufficient sensitivity. We have found a variety of bacterial DNA from various species, and whether these bacterial species are important to clinical pathogenesis remains largely unknown, since these patients were managed as "culture-negative", and no antibiotics were given to any of these patients. The bacteria identified by PCR/sequencing using 16S rDNA primers are diverse and many of them are known pathogens in human diseases (Table 1). Given the known low yield of the automated blood culture method, more sensitive methods of bacterial detection such as PCR/sequencing and more sensitive culture methods may improve patient care.

The recent description of bacterial DNA translocation is an interesting concept in CD and inflammatory bowel disease (IBD) [20,21]. It is also interesting that common pathogenic bacteria such as enterobacteria, Staphylococci, and Streptococci, etc., cannot be cultured from the blood of IBD patients [20,21]. During the process of "bacterial translocation", the epithelial barrier of intestinal mucosa is defective and the intestinal bacteria will travel through the barrier to the blood circulation and interact with inflammatory cells within the lamina propria or submucosa. The best example of bacterial translocation through epithelial cells and the lymphatic system is *Salmonella typhi* through cystic fibrosis transmembrane conductance regulator (CFTR) by the epithelial cell internalization [22,23,24]. However, bacterial "naked DNA" translocation is unknown, and the bacterial proteins in the blood cannot be demonstrated unless specific antibodies against bacterial proteins are employed. The fact that up to 20-50% CD patients are positive for anti-ompC antibody indicates a host specific response to ompC of *E. coli* [25]. The presence of bacterial DNA within the blood circulation is most likely a bacterial infection based on our study of MAP culture, and more studies are necessary to elucidate the mechanism of this peculiar pattern of growth of MAP, MAH and other medically important bacteria.

## Financial Disclosure

None.

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