The Paratuberculosis Newsletter

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Notes from the Editor

Is your passport checked and ready? Have you registered? The 10ICP in Minnesota is just around the corner. The organisers have reported that many abstracts have been submitted. I have been to the last four ICPs, and this year is no different. I am excited and looking forward to meeting old friends, making new friends, discussing paratuberculosis issues and hearing about what other ParaTB enthusiasts around the world are doing. So, if you are in the paratuberculosis business, this is the place to be. And if you have not already registered, check out http://www.cvm.umn.edu/outreach/events/icp/home.html.

Since the last newsletter, a few interesting contributions have made there way into this issue. As always, your contributions are welcome, and I would like to encourage you to provide information so that other members get to know what you are doing or what has been done in your country.

If you have read ParaTB articles in German, Dutch, Spanish, or any other language, and you think the articles include interesting findings, please translate it to English and submit it to me as your contribution. This would be a great way of making information accessible to everyone. For example, if some of the older paratuberculosis literature, originally written in German was translated. Have you ever read the original paper by Johne and Frothingham from 1895? This and a few other articles available here: http://www.ihh.kvl.dk/htm/ssn/history.htm. Please note that they are only in German. You could provide a service to your peers if you help translate one of these papers. It would then be published in The Paratuberculosis Newsletter, recognising also the translator. Let me know if you wish to translate a paper, and I will assist you and coordinate if more people have the same idea.

Remember that deadline for next issue is just after the 10ICP. So, you may consider submitting something you experienced at 10ICP for the newsletter or provide an anecdote from the colloquium along with some pictures.
See you at 10ICP!

Søren Saxmose Nielsen
Editor

DEADLINE FOR NEXT ISSUE: August 15, 2009.

All contributions should be sent to ssn@life.ku.dk

1. IAP Business

10ICP Awards

Once more, as we are getting close to the Colloquium dates, the IAP has completed the process of recognising and supporting some of its members. This year, all categories of awards have had candidates and therefore we have a total of 14 colleagues that will attend the 10 ICP supported by the IAP.

Emeritus awards
Two awards have been granted in this category. The awards will be announced at the Colloquium.

Richard S. Merkal Memorial awards
Again two young members have been selected from a total of 7 applications on the basis of the special interest of the papers they are going to present during the 10 ICP. The winners are: **Wiebren Santema** from The Netherlands and **Franziska Gierke** from Germany.

Helping Hand awards
For the third time these awards that were created to allow researchers from countries with lower income to attend the next Paratuberculosis Colloquium have been granted. It has been a difficult task to evaluate the 18 applications and to select the 10 that would better fulfil the goals of the call. Finally, the IAP Member Recognition and Support Committee has granted a Helping Hand award to the following persons:

J. Fernandez (Colombia)
J. Sohal (India)
K. G. Tirumurugan (India)
S. K. Munjal (India)
M. A. Salgado (Chile)
M. Pradenas (Chile)
V. N. Ngwa (Cameroon)
G. Snel (Brazil)
P. Singh (India)
B. S. Bercht (Brazil)

All these awards include free 10 ICP registration, free IAP Membership for 2009 and 2010, and a fixed amount for travel expenses as well as a certificate of their success in the corresponding call.

Congratulations to the winners and thanks to all for participating.

2. Short scientific reports

Investigating the interactions between Luciferase bacteriophages and MAP bacteria

Susan McCusker and Lucy M Mutharia

Luciferase reporter phage-based assays can be used as alternatives to culture-based assays because they reduce the time-to-detection of viable cells especially those of the slowly growing bacteria such as *Mycobacterium avium* subsp. *paratuberculosis* or MAP. LRP have also been used in antibiotic sensitivity screening assays. LRP are engineered to carry a lux-gene encoding for the enzyme luciferase which in the presence of the substrate catalyses production of bioluminescence. Because phage only replicate inside a viable host cell, luciferase production and therefore bioluminescence is only detected when a sample contains live cells of the target host.

We used electron microscopy to study the interaction between MAP, the mycobacteriophage TM4 and luciferase reporter phage (LRPs) phAE85, phAE39 and phAE40 (Jacobs et al., 1993; Carriere et al., 1997). Assay factors affecting phage infection of MAP cells and the detection of luciferase bioluminescence were also examined. All phages were propagated on *M. smegmatis* mc^2155. MAP cultured at 37°C in detergent free-Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) supplement, 0.2% glycerol, 2 mg/L Mycobactin J, 100 U/mL Penicillin G and 50 mg/L chloramphenicol. Logarithmic phage cultures (OD_{600nm} of 0.6 – 0.8) were harvested, the cell pellets washed twice and re-suspended in Middlebrook 7H9 broth (containing 0.2% glycerol and OADC) and incubated for 3h before infection with TM4 or LRP phAE85 at an MOI =100. After incubation (3 h at 37°C) a 100 µL volume of the bacteria-phage suspension was aliquoted onto a piece of parafilm and a formvar/carbon coated copper grid was floated on top for 5 min. The grids were blotted dry then floated on a drop of 5% uranyl acetate for 3 min and again blotted dry. The negatively stained preparations were examined by transmission electron microscopy.

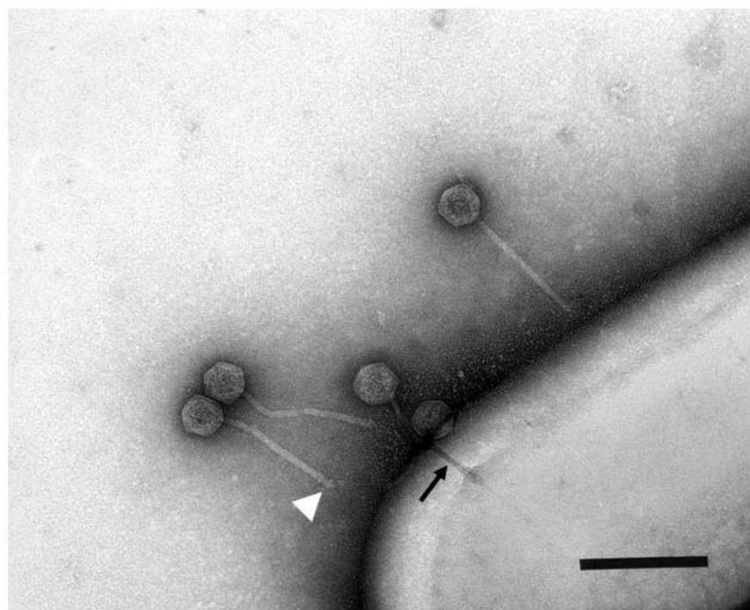

Fig. 1. Negatively stained electron micrograph of MAP ATCC 19698T infection by mycobacteriophage TM4 at an MOI = 100. The long phage tails appeared to have a “bulb-like” structure at their terminus (white arrowhead). (Marker = 200nm)

TM4 phage particles were observed to have isometric heads and tails (Fig 1). No tail fibres were visible but in some preparations a bulb-like structure was observed at the end of the phage tail (Fig. 1, white arrowhead). In several micrographs the phage tail appeared to 'penetrate' (Fig 1, black arrow) MAP bacterial cell wall and phage were clearly bound to the bacterial cell surface. Many studies have utilized phage inactivation by saccharides as a preliminary step in determining the location or identity of the phage receptor (Valyasevi et. al., 1990; Quiberoni et. al., 2000; Deveau et. al., 2002). In an effort to elucidate the phage receptor for TM4 and its progeny, phage inhibition of saccharides was examined. Sugars that inhibited phage infection of MAP cells abrogated production of luciferase-catalyzed bioluminescence or inhibited phage binding to MAP surfaces as viewed under electron microscopy. L-arabinose at concentrations of 100 and 250 mM inhibited by 62% and 76% respectively LRP phAE85 infection of MAP ATCC19698. D-arabinose, D-mannose, D-glucose and trehalose had no effect on phage-bacterium interactions. These results suggest that phage TM4 and its progeny may adsorb to a MAP cell wall component containing L-arabinose. Lastly, magnetic bead-capture of MAP cells decreased assay sensitivity by 10- to - 100-fold when compared to controls. The magnetic bead matrix interfered with the detection of bioluminescence.

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4. Comments & Opinions

Testing bovine milk for the presence of antibodies to *Mycobacterium avium* subspecies *paratuberculosis*

Gilles R. G. Monif

In partial response to the growing concern and scientific data that *Mycobacterium avium* subspecies *paratuberculosis* by accessing the human food supply may constitute a hazard to public health, the U.S. Johne's Disease Strategic Planning Subcommittee of the U.S. Animal Health Association proposed in its focus research efforts on control and prevention of Johne's disease that bulk tank testing be done using quantitative ELISA milk testing (Schwartz , 2008)). Earlier in 2008, the Animal Health Services of The Netherlands added testing of milk using commercial or in-house ELISA tests to its Proficiency Testing Schema /Ring Trials (BioChek. UK Ltd.: Announcement Ring Trial *Mycobacterium avium* subsp. *paratuberculosis* in serum and milk 2008. Letter of June 2008).

The purpose of Map milk ELISA testing is not clear. Map milk ELISA testing is not done to confirm the diagnosis of Map infection. IgG immunoglobulin secreted into milk represents only a fraction of the corresponding serum levels. Consequently, the titer identified in milk will be lower than that present in serum. Lombard et al evaluated 6,349 milk samples and found only a moderate agreement (kappa value of 0.50) between milk and serum ELISA results (Lombard et al., 2006).

The IDEXX and Prionic Map ELISA tests respective thresholds for determining a specimen to be either suspicious or positive are arbitrarily elevated in order to achieve a strong positive predictive value to the ultimate development of Johne's disease. As such, their threshold values further mask the ability of milk Map ELISA tests to determine if an animal is infected with Map. Only animals with a high IDEXX or Prionic Map ELISA tests will have test confirmation in milk.

When it comes to acting for the potential public good, testing of milk for the presence of Map antibodies is tangential to basic issue of whether or not pathogenic mycobacteria are present in a given sample of milk. The presence of Map in milk is primarily demonstrated by culture or by DNA identification using polymerase chain reaction (PCR) Map primers.

Pinedo et al. (2008) have demonstrated serum Map ELISA values have a poor correlation with the presence of Map in milk. Cows testing positive by milk PCR had negative and inconclusive ELISA results in 23.5% and 11.8% of the cases, respectively. Other than using quantifying levels of Map ELISA antibodies for bulk milk as an insensitive herd screening test for monitoring herd infection level, the test does not safe guarding the human food supply at the bulk milk level per the Rio Declaration for food safety.

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Mycobacterium paratuberculosis as an environmental organism

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Having watched from the sidelines the progress made on *M. paratuberculosis*[1] /Johne's Disease/Crohn's Disease over the last 15-years, I am totally dismayed at the misdirection the field has taken. *M. paratuberculosis* is NOT an environmental organism.

Maybe it is the willingness of researchers in the field to blindly accept others' results and interpretations; or the lack of understanding of general microbiology and infectious disease; or the misdirected opinion that PCR is infallible and gospel; or the lack of understanding and awareness of almost 150 years of past research efforts and results; or maybe some other reason the field has chosen its' erroneous direction. The actual reason(s) is not important; the impact, however, is.

It is a misnomer that old dogs cannot be taught new tricks; rather, it is difficult to break old habits. Like an old dog, it is going to be difficult to correct the erroneous interpretations and misdirection the field has taken. And you will not appreciate the damage that has been done until you try to take your results and interpretations outside your safe closed "nitch" of paratuberculous researchers.

So how did you guys determine that *M. paratuberculosis* is an environmental organism and why is this conclusion erroneous and damaging to the field?

It all starts and dates back to the effort to detect *M. paratuberculosis* in Crohn's disease tissues. Initial results, which only showed about 30% positive, was not satisfactory to the investigators and so they "tweaked" the system to improve sensitivity and thereby developed a PCR method that detected IS*900* in over 80% of Crohn's disease patients. The only problem was that control samples were now also positive (albeit not as many as the test samples). The only "logical" conclusion was that *M. paratuberculosis* is widespread in the environment and that everyone is exposed to it. There could, of course, be no other logical explanation.

From this small beginning, *M. paratuberculosis* (or rather IS*900*) is found in river water, milk, soil, and just about every other product that has to date been tested. Nothing is free of IS*900* and *M. paratuberculosis* becomes another ubiquitous environmental mycobacterial species.

Researchers begin to develop a host of novel and imaginative explanations, contrary to conventional wisdom and principles of infectious disease and microbiology, to explain how an environmental organism could be a primary pathogen and cause of human disease.

The hypothesis that some cases of Crohn's disease are caused by *M. paratuberculosis* is expanded to include ulcerative colitis, indeterminative colitis, and most recently irritable bowel disease. And some people wonder why the hypothesis that some cases of Crohn's Disease is caused by *M. paratuberculosis* is losing ground? In 1984, Alan Cantwell published his work "AIDS. The Mystery & the Solution" (Aries Rising Press, Los Angeles), claiming that AIDS is not caused by a virus but rather bacteria. I am just waiting for the day when *M. paratuberculosis* is proclaimed the real cause of AIDS. Why not, it seems to cause everything else? IS*900* data would not lie. Sound ridiculous? Is it any more ridiculous than claiming that *M. paratuberculosis* causes lymphadenitis simply because IS*900* was detected in the tissues?

Environmental bacteria are not primary causes of disease; they only cause disease in immunocompromised individuals, i.e., they are opportunistic organisms. Environmental microorganisms have the ability to replicate in the environment; otherwise they would be diluted out in the environment to levels below infection thresholds. This is a simple scientific

[1]The accepted nomenclature of the species is *Mycobacterium avium* subspecies *paratuberculosis*; however, the author rejects this classification and, under the rules of the IWGMT and the ICSP, the author adopts this species' original nomenclature in public protest of its reclassification.

fact which is taught in Microbiology 101. If such were not the case, we would all be dead by natural selection. Maybe most ruminants are immunocompromised or have some underlying infection/defect that makes them susceptible to infection with *M. paratuberculosis*? I don't know? You tell me, it is your theory.

If you were to trace a bacterial pathogen, let's say *M. tuberculosis* for example, we would find the highest concentration of the organism near the source of infection with less bacteria the farther we move away from the source. The organism gets diluted out. On the other hand, an environmental organism, such as *M. avium*, the opposite is true. We find more bacteria the farther away we move from the initial source because the organism is replicating. Which scenario sounds most like *M. paratuberculosis*?

Is there any evidence that *M. paratuberculosis* can replicate in the environment? No. In fact, data suggests the opposite. Does anyone remember what mycobactin is, it's function, and how it affects replication? From a pure biological perspective, *M. paratuberculosis* cannot replicate in the environment.

Like other life forms, mycobacteria require free iron for its metabolic processes. However, free iron rarely exists in nature and mechanisms must be developed to sequester and make that iron available to the organism. In mycobacteria, that iron chelator is mycobactin, a complex siderophore within the mycobacterial cell wall that has a very high affinity to bind iron. It competes with transferrin, lactoferrin, ferritin, and other iron chelating agents for ferric iron. In *M. paratuberculosis*, the mycobactin molecule is defective and has a low iron affinity. Thus, in order to grow *M. paratuberculosis* in vitro, either an exogenous source of mycobactin must be supplied or the organism must be grown under high iron conditions.

This requirement for an exogenous source of mycobactin is, in many respects, a laboratory artifact. Within the confines of *M. paratuberculosis*' preferred habitat, the macrophage, the low pH disassociates iron from its chelators, and thus, free ferric iron becomes readily available and mycobactin serves no function. A strict intracellular pathogen really has no use for mycobactin or any other iron-chelating agent as it is not functional intracellularly. Anyone that has ever harvested mycobactin has observed this first hand – mycobactin is removed from the column (bound to iron) by simply washing the column in acid.

However, outside its preferred environment (a living host), *M. paratuberculosis* has no available means of sequestering iron and without iron, it cannot grow. Thus, based solely on mycobactin –dependency, *M. paratuberculosis* is incapable of environmental replication, cannot be widespread in the environment, and must be a strict intracellular pathogen. This is further supported by the host of papers published in the past related to the survivability of *M. paratuberculosis* in the environment. No study has ever documented environmental growth, only survivability, and this is supported biologically. The only basis of this environmental claim is PCR.

So, if *M. paratuberculosis* cannot replicate in the environment, how does this relate to the IS*900* PCR results suggesting widespread distribution? Let's look at a couple of clearly erroneous (or at least "hard to believe") studies.

M. paratuberculosis (IS*900*) has been detected in river water downstream of cattle operations (the Thames River in England for example). We are not talking about a little creek where infected cattle routinely go and defecate, but rather, a major river way. I am not going to do the actual math, but consider the dilution effect involved in this river and the number of infected cattle (and infection level) required to attain detectable levels of *M. paratuberculosis*. It reminds me of the way Cornell University used to disinfect their infectious disease animal housing units – they used to simple run a sprinkler system for a couple of days. Unless we erroneously accept that *M. paratuberculosis* readily replicates in the environment, it would be mathematically impossible to realistically detect *M. paratuberculosis* in large bodies of water.

Other mathematically impossible studies are equally erroneous. *M. paratuberculosis* (IS*900*) has been detected in retail milk samples. It is not really an issue of its detection that raises eyebrows, but rather the level of alleged detection. In one of the studies originally reported, by using a semi-quantitative PCR, it was determined that there were approximately

1000 organisms per ml of milk. Now, with only limited knowledge of how milk is processed, the dilution effects, somatic cell count thresholds for Grade A milk, etc., it would seem mathematically impossible to attain such high levels in retail milk.

This leads us only to now suggest that *M. paratuberculosis* is replicating in milk as it allegedly does in the environment. It would be the only way to explain such high concentrations. Considering that milk is very low in iron and abundant with the iron-chelator lactoferrin (both considered natural bacterial inhibitors), and the amount of time from cow to retail, *M. paratuberculosis* would need to grow faster than *E. coli* in the absence of iron and low temperature. Either this is impossible or maybe the best growth medium for M. paratuberculosis is milk under refrigeration.

There are many other examples of impossible (or highly improbable) results that could be discussed herein, but the above examples should be sufficient to illustrate that something is wrong, terribly wrong.

So, where does that leave *M. paratuberculosis* as an environmental organism? Either you believe, contrary to general wisdom and fact, that *M. paratuberculosis* is capable of environmental replication and is an opportunistic pathogen, or there is something else that explains the recent data. That something else can only be IS*900*.

If IS900 is the problem or the reason for these results, there are only 2 possibilities: IS*900* is NOT species-specific and/or the methods employed in its detection render it non-species-specific. Whatever happened to the age-old principle that increasing sensitivity diminishes specificity? Does this rule not apply to PCR? Most researchers in the field will, behind the scenes, question the specificity of IS*900* (at least within the parameters of the methods used) but fail to take it further, make an effort to determine what the "problem" might be, or publically raise the issue. The time has come to address these issues, to stop "sweeping them under the carpet", and seize the reporting of erroneous results.

Does it really matter? Well, that really depends on your objectives. To understand the impact of these findings, you need to understand some of the politics behind the history of *M. paratuberculosis*. We all know that science and politics do not make a good match, but you are a fool if you do not think that politics plays a major role in all aspects of science, either to its benefit or detriment.

First, understand and accept that paratuberculosis researchers (and veterinary-related research in general) are peons in the scope of science. USDA is dwarfed by NIH and NSF; NADC is dwarfed to CDC. The powers (and politics) of all infectious diseases are controlled by the microbiologists (NIAID).

In the late 1980's, there was a great deal of attention devoted to *M. paratuberculosis* as a zoonotic agent, ie., possible cause of Crohn's disease. But *M. paratuberculosis* was a poorly defined species that needed proper classification. The Working Group on Mycobacterial Taxonomy (IWGMT), a subsidiary of the International Society of Systemic Bacteriology, was being pressured into looking at *M. paratuberculosis*. This put the IWGMT into a dilemma. The IWGMT generally works by sending a blinded species to its members and having each member individually identify and classify the species. By this method, a complete taxonomic matrix considering all parameters would be developed.

As expressed to me by Larry Wayne, then head of the IWGMT, there was no way to undertake this task. If *M. paratuberculosis* were sent through the IWGMT, its' members would likely report back an unculturable acid-fact organism. If he gave them a hint, e.g., add mycobatin to the culture medium, the entire process would not be blinded and void. The solution? Have Marie Thorel, a member of the IWGMT, do a genetic matrix analysis of *M. paratuberculosis*, ignoring all other properties, and propose the taxonomic classification of *M. paratuberculosis* based on a genetic matrix. The results were not unexpected and *M. paratuberculosis* was reclassified as *M. avium* subspecies *paratuberculosis*. Problem solved: *M. paratuberculosis* becomes a member of the opportunistic environmental mycobacteria with little to no clinical significance and can now be ignored by the powers to be. The Veterinary Profession embraced this new classification (I think I am the only one that has and continues to reject this classification[1]) and *M. paratuberculosis* was thrust into the classification as an opportunistic organism. Other than veterinary books discussing Johne's

Disease, *M. paratuberculosis* can be ignored by Microbiology Texts as anything relevant would be covered by the general *M. avium* discussions.

The recent embrace of the widespread distribution of *M. paratuberculosis* (albeit likely erroneous) served to corroborate the IWGMT's contention that M. paratuberculosis is just another opportunistic *M. avium* species of no real clinical (human) significance.

If you believe that *M. paratuberculosis* may be the cause of some cases of Crohn's Disease (or associated in with any human disease), you will not convince anyone (within the powers to be) that *M. paratuberculosis* causes any disease in humans as a primary pathogen when it is, by definition and corroborated by IS*900* PCR, a widely distributed environmental organism that is, again by definition, an opportunistic pathogen. It is not, and cannot be, by definition, the primary cause of disease.

You have successfully destroyed or, at the very least severely compromised, any realistic hopes of convincing anyone (other than yourselves) that *M. paratuberculosis* causes any disease in humans. The "great white hope" for paratuberculosis research and interest has been flushed down the toilet.

The choice is yours. You can dig your hole deeper and continue down your present path. Or, you can start to dig your way out. You could start by taking a hard look at your IS*900* PCR and figure out how to make it coincide with basic microbiological concepts and principles. You cannot break all the rules and hope to win in the end.

Unfortunately, this entire article only addresses one of the many serious and detrimental directions paratuberculosis research has taken (particularly as related to human disease). You cannot make up your own rules, principles, and concepts in the hopes of gaining credibility and/or acceptance. It just doesn't work that way. It may be time to sit back and take a hard look at yourself.

The criticisms that have been lodged against the field in the mid-1990's not only still exist today, but have been unequivocally exacerbated. The National Institutes of Health, specifically the National Institute of Allergy and Infectious Disease (NIAID) raised 2 general criticisms of the paratuberculosis / Crohn's disease field: (1) that the researchers in the field had lost objectivity; and (2) that no organism has ever been looked for as thoroughly as *M. paratuberculosis* (related to controls and baseline of comparison). Every criticism since then can really be related back, directly or indirectly, to these 2 criticisms that have never been addressed.

When is the last time you sat down with a microbiologist or gastroenterologist and really listened to their skepticism and criticisms and understood them? Or, did you devote your time to trying to convince the person why their criticism was wrong and, through your own rules, principles, and concepts, try to convince them you were right? Does criticism #1 above ring a bell?

But other criticisms, misdirection, alienation, and erroneous adopted concepts will have to wait for another "venting" session.

5. Paratuberculosis Calendar

Please report to Søren Nielsen (ssn@life.ku.dk) should you have knowledge of any events that you find relevant to include in the calendar.

2009

August 9-13, 2009. 10th International Colloquium on Paratuberculosis, St. Paul/ Minneapolis, Minnesota, USA (http://www.cvm.umn.edu/outreach/events/icp/home.html).

August 10-14, 2009. 12th International Symposium on Veterinary Epidemiology and Economics. Durban, South Africa (http://www.isvee12.co.za).

August 25-28, 2009. *M. bovis* V Conference, Wellington, New Zealand (http://www.mbovisconference.org/)

6. List of Recent Publications

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