The Paratuberculosis Newsletter

March 2010

**An official publication of the
International Association for Paratuberculosis**

Table of Contents

Notes from the Editor

The year 2010 has started well! – at least from the view as the editor of this newsletter. I think this edition has the most contributions in the past few years, and I truly appreciate that. However, more contributions are most welcome!

The IAP Business section with the information of the resignation of our webmaster for 12 years, Alan Kennedy. A piece of information received much to my dismay, because Alan has helped significantly with the tasks I have carried out for the IAP. He may return some day? – at least that has been seen before, as exemplified by the announced return of past president Rod Chiodini.

The newsletter is not just about those who leave and those who joins or re-joins. It is also about providing information about ongoing research, it is about providing good advice and providing food-for-thought. I was happy to receive a summary of good pieces of advice from Irene Grant and Antonio Foddai. Basically, the listed "How to avoid all common the mistakes", which is always nice lists to have. Do you have a list of solutions to commonly made mistakes? – a piece of good advice? Please share them.

Opinions are also welcome. Gilles Monif touches on a topic which I enjoy: The probability diagnosis. Will detection of MAP infections ever be other than a probability? Should we not keep it like that, without announcing a given test as a gold standard? Is there a gold standard, even a silver standard, or will these perceived standards merely act as lead, which pulls us to the bottom of the sea?

If you have an opinion, do not forget to express it in this newsletter. You do not have to share mine

DEADLINE FOR NEXT ISSUE: May 15, 2010.

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

Søren Saxmose Nielsen
Editor

1. IAP Business

President's Word

Dear IAP fellow members,

In these early weeks of the year I would like to greet you once more as the first 2010 Paratuberculosis Newsletter issue sees the light. This is a between-Colloquia year in which we still have fresh the good times during the 10th ICP in Minneapolis while we are already looking forward to attend the 11th ICP in Sidney. I thank Scott Wells and the group that worked so hard and successfully to make enjoyable our time in Minneapolis. As you already know, we just got that meeting scientific contents records available in the form of the 10th ICP Proceedings. They bring two novelties. The first is the timing that has been the shorter for any colloquia up to now. The second is that given the agreements with the University of Minnesota and the support of the Governing Board, the Proceedings go fully public from their first issuing. This is the natural updating of the IAP standing in relationship to one of its goals: the spread of knowledge on paratuberculosis. Indeed, the growing of the Open Access publication policies where those doing the research with public funding also pay for making it publicly available needed an answer from the IAP. This is made even more pressing an issue since we are in a time in which recording and distribution technologies have taken off and have made some of the 10th ICP presentations available in the web just a few days after making them. This thinking has led the IAP to implement the By-Laws mandate to edit the Colloquim proceedings in a simplified style in order to shorten the time to publication and to reduce the amount of work involved. We owe this mainly to the dedication of Soren Nielsen as the IAP Editor-in-Chief and that of Scott Wells as Chairman of the 10th ICP Organizing Committee as well as to the technical support of Alan Kennedy.

Alan Kennedy was the person who, being interested in paratuberculosis as an agent of Crohn's disease and having the technical knowledge and skills to work it out, designed the IAP website, set it up and has been running it as the webmaster for so many years. Now he has decided that he must move forward to other goals in his life and has resigned as the IAP site webmaster. I want to acknowledge here his invaluable efforts and the time with what he has gifted the IAP beyond professional duty. The new webmaster has a though challenge to build on that and to reach similar standards.

I cannot finish without sharing with you the expectations for the next colloquium that Richard Whittington and his group is endeavouring to have ready for the in Sidney. In addition to the opportunity to visit wonderful Australia once more and meeting with the old friends, I am sure the Colloquium will widely fulfil its goals of sharing the latest observations, hypotheses and models on paratuberculosis and thus make us feel first hand the thrill of playing a part in the progress of Science.

Ramon A. Juste
President of the IAP

International Association for Paratuberculosis

Financial Report—Final 2009 (1/1/2009 to 12/31/2009)

	Checking	Savings	CD	Total
Opening balance (1/1/09)	$4,2639.29	$30,932.81	$54.524.53	$ 89,693.63
Q1 Closing balance (3/31/09)	$ 8,811.78	$29,450.46	$54,926.62	$ 93,188.86
Q2 Closing balance (6/30/09)	$32,635.83	$29,461.35	$55,338.70	$117,435.88
Q3 Closing balance (9/30/09)	$16,753.39	$25,608.45	$55,776.99	$ 98,138.83
2009 Closing balance	$15,921.81	$22,431.32	$56,221.76	$ 94,574.89

Receipts

	Dues	Book Sales	Interest	Other	Total Receipts
Q1	$5450.00	$220.00	$419.83		$ 6,089.83
Q2	$3250.00		$422.97	9ICP $ 20,754.51	$24,427.48
Q3		$ 700.00	$ 95.00	$445.39	$ 1,240.39
Q4			$449.67		$ 449.67
2009 Total					$32,207.37

Expenses

	Credit card processing fees	Other	Total Expenses
Q1	$307.01	Open Journ Sys $ 787.50	$ 2,594.60
		Web Master	$ 1,500.09
Q2	$180.46		$ 180.46
Q3	$111.17	10ICP Stipends/Awards $ 17,958.99	
		10ICP (plaques, meal, travel) $ 2,467.28	$ 20,537.44
Q4	$ 31.58	10ICP travel, awards$ 3,982.03	$ 4,013.61
2009 Total			$ 27,326.11

Net income

Q1	$ 3,495.23
Q2	$ 24,247.02
Q3	$ (19,297.05)
Q4	$ (3,563.94)
2009 Total	$ 4,881.26

-submitted 1/25/10, Raymond W. Sweeney, VMD; Secretary-Treasurer

2. Short Scientific Reports

The true prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in Cypriot small ruminants

M. Liapi[a], L. Leontides[b], P. Kostoulas[b], G. Botsaris[c], Y. Iacovou[a], C. Rees[c], K. Georgiou[a], G. C. Smith[d], D.C. Naseby[e]

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Important findings
- ➢ MAP-infection is widely spread among the small ruminants on the island.
- ➢ Two out of three sheep flocks and one every two goat flocks have at least a MAP-infected animal.
- ➢ Within these infected flocks the prevalence is, on average, close to 25%.
- ➢ From a control point of view, it is encouraging that one of three sheep flocks and one of two goat flocks may not have infected animals.

In September 2005, a serological survey to estimate the true prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) of sheep and goats, older than 2 years, was undertaken in the region effectively controlled by the Government of the Republic of Cyprus.

From the total population of 339371 sheep and goats reared in 3878 flocks, after applying a combination of stratified and cluster sampling, sera were collected from a random sample of 8011 animals (3429 sheep and 4582 goats) from 83 flocks, non-vaccinated against MAP. They were examined for antibodies with the Pourquier® paratuberculosis antibody screening ELISA.

The true prevalence of MAP-infection was calculated, separately in sheep and goats, with Bayesian models that accounted for the misclassification of animals because of the imperfect accuracy of the serologic test.

The mean true prevalence of infected sheep and goats was 15 and 11.1%, respectively. There was at least an infected sheep in 60.8% (95% credible interval: 42.3; 78.8%) and at least an infected goat in 48.6% (30.4; 68.5%) of the flocks. In the infected flocks, the mean within flock true prevalence of infection in sheep and goats was 24.6 (16.3; 33.3%) and 23.1% (15.5; 33.6%), respectively.

Surveillance of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds

Maarten F. Weber

GD Animal Health Service, P.O. Box 9, 7400 AA Deventer, The Netherlands

Summary of PhD thesis
In this thesis, the potential for improvements in surveillance of *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection and paratuberculosis in dairy herds was investigated, leading to a reduction in surveillance costs whilst continuing to meet specific quality targets. In particular, differentiation of surveillance strategies to accommodate the aims and needs of various groups of dairy farmers was studied.

A stochastic simulation model (JohneSSim) was used to identify cost-effective test schemes for a surveillance programme for certified 'Map-free' dairy herds and a bulk milk quality assurance programme for paratuberculosis (BMQAP) in dairy herds. In addition, field data were analysed to obtain information useful to the improvement of surveillance systems for Map. This included analyses of cattle transfers between certified 'Map-free' herds, an evaluation of a serum-ELISA as tool in the surveillance of clinical paratuberculosis, and an analysis of the age at which cattle become infectious.

Based on the results of the simulations as well as the analyses of field data, recommendations were made on the design of the Dutch surveillance programme for 'Map-free' herds and a BMQAP. The analyses of field data showed that the contact structure of cattle transfers between 'Map-free' herds was not random but underdispersed, which adds substantially to the guarantees provided by the 'Map-free' status. Furthermore, it was shown that a serum-ELISA is useful to confirm a clinical presumptive diagnosis in the surveillance of clinical paratuberculosis. Finally, it was shown that a substantial proportion of cattle in infected herds become infectious before two years of age.

The thesis is concluded by a discussion on the application of the study results, effects of surveillance of Map and paratuberculosis on the interests of consumers, dairy processing industry and dairy farmers, and recommendations on future developments and research regarding the surveillance of Map in dairy herds.

As of January 2010, the Dutch dairy industries require all dairy herds delivering milk to their factories to participate in either the BMQAP or the surveillance programme for 'Map-free' dairy herds that were studied in this thesis.

A limited number of copies of this thesis is available for interested IAP members. If your wish to receive a copy, please contact the author (m.weber@gddeventer.com)

How to modify the commercial *FASTPlaqueTB*[TM] phage amplification assay protocol to detect and accurately enumerate viable MAP in milk

Irene R. Grant and Antonio Foddai

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The *FASTPlaque*TB[TM] phage amplification assay manufactured by Biotec Laboratories Limited, Ipswich, UK (now part of Lab21 Limited, Cambridge, UK) is designed for the detection of *Mycobacterium tuberculosis* from human sputum samples. Stanley et al. (2007) were the first to demonstrate that this commercially available phage assay can also be used for the detection of MAP in milk. We have recently published two further papers in relation to using the commercially available *FASTPlaqueTB*[TM] assay to detect and enumerate viable MAP in milk (Foddai et al. 2009, Foddai et al. 2010). The first of these papers described optimisation of the conditions of the commercial *FASTPlaque*TB[TM] assay to allow more accurate enumeration of MAP, i.e. towards achieving 100% correlation between colony counts (CFU/ml) and plaque counts (PFU/ml). The subsequent paper described a study demonstrating that the optimised phage assay could be used in place of culture on Herrold's egg yolk medium (HEYM) to enumerate surviving MAP after heat treatment. In both these studies we were able to use in-house prepared D29 mycobacteriophage and *Mycobacterium smegmatis* sensor cells, which other laboratories will not have access to. Therefore, in order to promote more widespread use of this rapid phage amplification assay for viable MAP within the IAP community, the purpose of this article is to explain how the standard *FASTPlaque*TB[TM] protocol, as described in the instructions accompanying the kit, can be modified with non-commercially prepared reagents. Unless the optimised conditions are used accurate enumeration of MAP will not be achieved and numbers of MAP present will be underestimated. Several studies have demonstrated that plaque counts can be 1-2 $\log_{10}$ less than corresponding colony counts when the standard phage assay conditions are used (Altic et al. 2007, De Buck et al. 2008, Donaghy et al. 2009, Foddai et al. 2009).

Our research (Foddai et al. 2009) demonstrated that the *FASTPlaque*TB[TM] assay conditions which needed to be optimised or changed to permit accurate enumeration of MAP were: calcium chloride concentration in the broth medium, overnight incubation of the MAP cells recovered from milk before phage addition, and extended incubation time before virucide treatment. Although an increase in PFU number can also be achieved by increasing the total incubation time before plating, care should be taken that this period is not longer than the replication time of the phage (shown by us to be approximately 210 minutes, but may vary depending on the state of the MAP calls in different experiments). The required modifications to the standard protocol and *FASTPlaque*TB[TM] Media plus broth are indicated in Table 1.

The following are some further hints and tips in light of our experience with the optimised phage assay to date:

- If using the optimised phage assay to test naturally infected milk samples it is important that milk samples are as fresh as possible (to ensure low background milk microflora) and have not been frozen and thawed (strange pellet obtained in our experience).
- In the case of naturally infected milk it is advisable to add NOA antibiotic supplement (available from Biotec Laboratories Limited, now Lab21) to the Media Plus (personal communication from Dr Cath Rees, University of Nottingham). NOA is a mixture of three

antibiotics designed to combat growth of background flora in sputum samples (Albert et al. 2007). It should be added to FPTB Medium Plus at the level indicated in accompanying instructions to combat overgrowth of sensor cell (*M. smegmatis*) lawn by contaminants, with possible masking of plaques leading to false negative results. Whilst NOA may not be the optimal antibiotic mixture to combat milk microorganisms it is preferable to no antibiotics at all.

- If background microflora overgrowth remains a problem even after NOA addition, leave out the overnight incubation at 37°C before commencing the phage assay. Plaque numbers will be reduced, i.e. MAP numbers underestimated, but presence/absence of potential MAP should still be indicated.

- It should be noted that any plaques obtained using the optimised phage assay in the absence of prior IMS (to achieve selective capture of MAP) could come from *Mycobacterium* spp. other than MAP. This is a consequence of the fact that the phage has a broader host range than MAP, *M. tuberculosis* and *M. smegmatis* (Rybniker et al. 2004). In this case it would be important to harvest a representative number of plaques and perform Plaque-PCR as described by Stanley et al. (2007) and Botsaris et al. (2010) to verify that the identity of the *Mycobacterium* sp. giving rise to each plaque is indeed MAP.

- NOA supplement is not required for studies with MAP broth cultures or MAP spiked UHT milk samples (as used by Foddai et al. 2010) because no background microflora exists.

We would encourage other MAP researchers to try out the *FASTPlaque*TB^{TM} assay with the above modifications. It is such a simple assay, but it has major advantages for detecting and enumerating <u>viable</u> MAP in milk in terms of speed (result within 48 h) and sensitivity compared to conventional culture which necessitates decontamination with 0.75% cetylpyridinium chloride for 5 h followed by culture on HEYM or in BACTEC or MGIT media for many weeks.

Any further queries regarding use of the modified *FASTPlaque*TB^{TM} assay should be directed to Dr Irene Grant (i.grant@qub.ac.uk).

Table 1. Modifications required to commercial FASTPlaqueTB assay to achieve accurate detection and enumeration of MAP in milk.

Required conditions for MAP detection	Modification to standard protocol
2 mM CaCl$_2$ in Media Plus	Amend FPTB Medium (supplied) after addition of FPTB growth supplement (supplied) by adding 200 µl of filter-sterilised 1 M CaCl$_2$ per 300 ml of Media Plus.
Overnight incubation of sample before phage addition	Centrifuge 50 ml milk, resuspend pellet in 1 ml amended FPTB Medium Plus (see above), and incubate at 37°C overnight in flip-top vials (supplied).
Virucide treatment after 2 h	Incubate sample for 2 h (not 1 h) after addition of Actiphage (supplied) before adding reconstituted Virusol (supplied).
Extra incubation before plating with sensor cells	After addition of 5 ml FP Media Plus to stop virucide, place samples back in incubator for a further 1 h (i.e. 3 hours total incubation time) before plating in FPTB agar (supplied).

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3. Comments & Opinions

After 15-year, I am back….

Rod Chiodini

Texas Tech University Health Sciences Center, Paul L. Foster School of Medicine, Department of Internal Medicine, Division of Infectious Disease, 4800 Alberta Avenue, El Paso, Texas 79905, USA

To the delight of some, dismay of some, and "who cares" by many, after a 15-year absence from the field, I have been recruited by Texas Tech University Health Sciences Center for the specific purpose of addressing the *M. paratuberculosis*-Crohn's disease issue, specifically causality.

This will be accomplished by going back to 1998 and finally addressing the National Institute of Allergy and Infectious Diseases (NIAID) suggestions and criticisms which to date have remained unaddressed and unanswered and hold the key to establishing a causal or incidental role of *M. paratuberculosis* in human disease. Association (mere detection) is not the same thing as causality, particularly when dealing with an agent that has never been conclusively shown to cause any disease in humans.

The specific questions to be addressed include:

1. Does *M. paratuberculosis* exist within a subpopulation of Crohn's disease patients at a higher frequency than other infectious agents?

    As noted by NIAID over 10-years ago, no infectious agent has ever been sought in the history of science with the same intensity and vigor as that which has been applied to *M. paratuberculosis*. Thus, there does not currently exist a background or framework on which the significance of *M. paratuberculosis* detection can be determined. If *M. paratuberculosis* has the environmental distribution as suggested by some studies, the increased detection rates in Crohn's disease (and ulcerative colitis) could easily be caused by antigen trapping within the disrupted intestinal architecture. That being the case, other environmental organisms and ubiquitous infectious agents should also be detected at a higher rate within diseased tissues. Science/Medicine is not absolute, it is relative, and without a comparative basis on which to determine relevance, the significance of *M. paratuberculosis* cannot be determined.

2. Does *M. paratuberculosis* exist in greater numbers within diseased tissues as compared to non-diseased?

    If *M. paratuberculosis* causes some cases of Crohn's disease, the number of organisms within the diseased tissues must be greater than the number of organisms detected in non-diseased tissues (controls) as well as normal tissue within the same host. If the actual number of organisms (colony forming units: CFU's) are greater in diseased as opposed to non-diseased tissues, the detection of *M. paratuberculosis* would be significant. Conversely, if the numbers of organisms present in diseased

tissues are not different than those found in non-diseased tissues, the presence of *M. paratuberculosis* is likely incidental.

3.   <u>Is the detection of *M. paratuberculosis* in diseased tissues a reproducible finding?</u>

To date, all efforts to detect *M. paratuberculosis* have been at single time points. If *M. paratuberculosis* causes some cases of Crohn's disease, the detection of *M. paratuberculosis* should be reproducible (within the limits of available technologies). At present, it is unknown if an individual is positive/negative today, what will the results be tomorrow? If results are not reproducible, then testing for *M. paratuberculosis* has no meaning or functionality (i.e., results are inconclusive and meaningless).

4.   <u>Can a subpopulation of *M. paratuberculosis* associated disease be identified?</u>

Assuming the above steps are true (i.e., *M. paratuberculosis* exists in patients at a significantly greater frequency as compared to other ubiquitous intestinal pathogens; the number of organisms is greater in diseased tissues as compared to non-diseased tissues; and the detection of *M. paratuberculosis* is a reproducible finding and not random), then there exists an objective method to define a subpopulation of Crohn's disease patients with *M. paratuberculosis*-associated disease.

Since *M. paratuberculosis* has not yet been shown to be the cause of any human disease, it is not important how many Crohn's disease patients may or may not have *M. paratuberculosis*- associated disease – the goal is to identify an objective population on which additional studies can be performed.

5.   <u>Can the role of *M. paratuberculosis* in Crohn's disease be defined?</u>

Once an objective subpopulation of Crohn's disease patients with *M. paratuberculosis*-associated disease can be identified, the opportunity will exist to specifically define the role, if any, of *M. paratuberculosis* in Crohn's disease. This will require monitoring the level of *M. paratuberculosis* infection (number of organisms/gm tissue) during treatment with antimicrobial agents. If disease improvement can be correlated with diminishing numbers of *M. paratuberculosis*, causality can be defined. Alternately, diminishing numbers of *M. paratuberculosis* in the absence of disease improvement would indicate an incidental role.

The proposed research program is designed to define the role of *M. paratuberculosis* in Crohn's disease and has an equal probability of dismissing the notion as it does in proving it. After 25-years, either outcome would be an advancement and blessing.

If you are interested in learning more about what we are doing and exactly how we will accomplish the goals and 5 steps defined above, visit us at The Crohn's Disease Initiative (www.thecrohnsdiseaseinitiative.com). Look forward to seeing you there.

When is *Mycobacterium avium* subspecies *paratuberculosis Mycobacterium avium* variant?
and
When is *Mycobacterium avium Mycobacterium avium* subspecies *paratuberculosis*?

Gilles R. G. Monif, M.D.

Mycobacterium avium subspecies *paratuberculosis* (Map) and *Mycobacterium avium* complex (Mac) have had sufficient differences to warrant taxonomic their identification as separate species. Initial data derived from 16rRNA sequencing and nucleic acid hybridization had been supportive of the current taxonomic classification (1-3).

What have been under appreciated are the biological similarities that speak of a common evolution tract (4). Using internal transcribed spacer of the DNA sequence, Frothingham postulated that the pathogenic mycobactereia had emerged through an evolutionary bottleneck (5).

The validity of the prevailing taxonomic assumptions of specificity is open to serious challenge.

In 1986, using immunodiffusion analysis, McIntyre and Sanford presented data inferring that Map as well as other mycobacterium is a variant of *Mycobacterium avium* (Ma) (6). In 1987, McFadden et al., using DNA probes to identify restriction-fragment-length polymerase similarly concluded that Map is likely a genomic variant from Ma (7). In 1988, Saxegaard and Baess, using DNA hybridization demonstrated a relationship between Ma and Map (3). In 1988, Thoresen and Saxegaard, using the Gen-Probe Diagnostic System directed against 16S rRNA, further strengthen the contention that Map is not genetically a homogenous species (8). The 16S rRNA, being considered an important phylogenetic and taxonomic indicator, could not differentiate Ma from Map. In 1990, Collins et al. using restriction endonuclease analysis and DNA hybridization demonstrated Map strain variability (9). In 1990, Whipple et al., using restriction-fragment-length polymorphism analysis, identified Map isolates that were not recognized by restriction endonuclease analysis (10).

Over the past two decades, fundamental conceptual errors have been propagated by United States Department of Agriculture (USDA) that have distorted the natural history of Map infection as opposed to that of disease.

The presumption that Map is a homogenous species resulted in the acceptance that the Linda strain was appropriate for genomic characterization. The Linda strain of Map was originally isolated from a girl with Crohn's disease. The decision to use a Map human vs. a bovine isolate disregarded the possibilities that between Ma and Map there might be pathogenic genomic variants and that the Linda strain might not be a centralist organism for the species. Presumption of homogeneity is also the primary reason that USDA mandated isolate specificity, thereby relegating Ma and Ma/Map variants into the category of environmental mycobacteria.

Industry has complied with USDA's mandate for specificity by basing current serological and organism confirmation tests on the Map's IS*900* insertional sequence. Presumably to protect itself from liability secondary to false-positive tests, the veterinary diagnostic industry has elected to set the threshold for positivity at a high level so as to be able to achieve a strong positive correlation between a positive test and the probability of ensuing clinically overt Johne's disease.

The IS*900* insertion sequence per se does not guarantee specificity (11-15)

The net result is a badly flawed epidemiological data base from which a coherent nation herd management schema is difficult to extract. Map and Crohn's disease are bounded in a yet to be determined degree of causality (16-22). What ever policy is forthcoming from USDA and/or its European counterparts will have to effectively minimize the entry of Map and other related genomic variants from entering the human food chain through milk and milk-related products.

If one is using serological tests to determination the possible addition of an animal to a dairy herd or zoo, the results, at best, are a partial answer. Flawed serological tests have allowed pathogenic clinical isolates to go undiagnosed and to function as added disseminators of infection and disease

If you are using IS*900* primers to identify a culture isolate as Map, the result are, at best, a statement of high probability. Unless you are using Gen-Probe's 16S rRNA or Allied Monitor's IS*1300* primers, you are missing Ma/Map variants.

Two decades have been wasted in which the wrong questions based upon flawed assumptions have been asked. But the real problem confronting a solution is inferred in a quote from Louis Pasteur:

"New revolutions of thought, even those imposed by scientific demonstration leave behind vanquished ones who do not easily forgive" – 1861.

To this thought, someone might add a partial quote from General Charles de Gaulle in which he spoke of "les mouton".

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4. Events

The Japanese Society for Paratuberculosis is planning to hold a Specialized session for "Immunology of bovine paratuberculosis" supported by OECD and 9IVIS organizing committee in University of Tokyo.

The meeting will be held in August 16, 2010, as a satellite meeting to 9IVIS in Tokyo (http://9th-ivis.jtbcom.co.jp/).

Information about the specialized paratuberculosis session can be found at: http://wwwsoc.nii.ac.jp/jsp3/pdf/OECDsymposium-E12SS.pdf

5. List of Recent Publications

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