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# Study on Bio-Incidence of Mycobacterium Avium Sub-Species Paratuberculosis in Diabetic Patients in Agra Region in India Using Molecular Assays

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ABSTRACT

In earlier times there were a hypothesis that the Mycobacterium avium paratuberculosis (MAP) is associated with the Crohn's diseases (CD) but now-a-days with the help of new molecular biology techniques it is proven that MAP is associated with CD. Novel susceptibility genes for CD have been found out with the help of genome wide association studies. Adaptive immunity works against and protect us from intra cellular pathogens, it may be M. tuberculosis infection. The bacteria of MAP causes CD in humans but according to some reports giving the antimycobacterial therapy failed to give a constant response in the patients suffering from CD.

## Introduction

Mycobacterium avium subspecies paratuberculosis is a obligate pathogenic bacteria [1]. It is extremely slow growing, gram positive, acid fast bacteria, multi species pathogen [2]. It is the cause of JD in animals leading to persistent diarrhea, weight loss and death and has also been associated with CD in human beings. It is also written as M. avium ssp. paratuberculosis.

***Scientific classification of MAP***

| | |
|---|---|
| Domain: | Bacteria |
| Phylum: | Actinobacteria |
| Class: | Actinobacteria |
| Order: | Corynebacteriales |
| Family: | Mycobacteriaceae |
| Genus: | Mycobacterium |
| Subgenus: | Mycobacterium avium complex |
| Species: | M. avium |
| Subspecies: | M. avium ssp. paratuberculosis. |

Milk is an important vehicle for the transmission of MAP in human beings from animals. MAP have the ability to escapes from standard pasteurization conditions [3]. MAP is heat resistant and it is able to segregate itself in WBCs, so it constantly present in milk of infected animals. Other than CD in human beings it is a strong candidate for 18 other disease, which are autoimmune disorders, some of them are Type 1 diabetes, rheumatoid arthritis, hashimoto's thyroiditis, autism, multiple sclerosis, blau syndrome, kawasaki disease, sarcoidosis. Accoding to some studies MAP shares genetic susceptibility with Type 1 diabetes (Rani et al., 2010). The genetic similarity between these two is SLC11A1 gene which codes for membrane protein of monocytes, macrophages, glutamic acid decarboxylase, heat shock protein 60 (Paccagnini et al., 2009, Dow and Sechi 2011,Yang et al., 2000).

In animals its transmission is through fecal-oral route, through contaminated milk or food products or through contaminated surface [4]. The infection of MAP is initially observed in the intestine, but after some time it will spread liver, kidney, utreus, spleen as well as in lymphatic system, we can isolate this bacteria from all these organs and their organ systems [5]. Infected animals shed this bacteria in their milk and feaces. There is not any effective vaccine or a cure of JD in ruminents and primates, but the efforts are done by researchers to develop a vaccine for JD but it is believed that Mycopar® contains inactivated MAP and an adjuvant which will help to some extent(Ganusov et al., 2015). An effective vaccine of MAP can decrease the chances of spreading of this disease, improve animal health.

The cell wall deficient cells are known as spheroplast which were subcultured and later they will results positive for Ziehl Neelsen staining and there were a possibility that the isolated organism is mycobacterium, which were confirmed by the DNA hybridisation and later on by Insertion seuence IS900 PCR [6].

MAP is the strong and recurring candidate for research and it is proven by many reasons which includes: It is the main cause of epidemic of chronic colitis in cattles and some other species including primates. The bacteria of MAP can be isolated from intestine and blood of infected CD patients. Immune system of our body can make anti bodies against this microbe and these are disease associated but for the curing of disease the antimycobacterial drug is used.

There are some mechanism which can stay the presence of pathogenic immunological response in the body are:

1. The presence of this microbe causes inflammation in the intestine.
2. When the natural florae of the gut goes out of balance.
3. The host genetic defects in comprising of commensal microbiota.
4. Defects in host immuno regulation.

The above mechanism increases the possibility of exposure of bacterial antigens to mucosal-T cells and they also changes the host's immune response to commensal bacteria. It is believed that MAP is a persistent pathogen in the gut of every CD patients and it is proved by detection of MAP bacteria in the blood of CD patients. But it could be a secondary phenomenon due to inability of macrophages to kill MAP in CD patients or it may be due to the increased gut permeability. Increased gut leakiness hypothesis is promoted by the presence and detection of some other bacteria in the CD patients when they are compared with control groups and it was also observed that there is lack of specificity of detection of MAP bacteria.

## History of MAP, Crohn's Disease and Johne's Disease

This area requires a lot of research but very less progress is done in the field of this bacteria.The important historical developments in the field of MAP, CD and JD are as below:

**1769:** In this year, a post mortem of 34 year old man is done who is suffering from abdominal pain, ulcers, inflammation in ileum and colon, diarrohea, large mesentric lymph nodes since last 14 years and the post mortem is performed by Morgagni. It was attributed that it was intestinal tuberculosis as the CD is not diagnosed till then [7].

**1813:** At Royal College of Physicians, Combe and Saunders found a case of thickening of ileum [8].

**1882:** Microscopic and macroscopic features of Crohn's Disease was described by Moore as he found the intestinal obstruction in a patient whose colostomy has been done [9].

**1905-1912:** Many surgeons came across the cases who are suffering from chronic inflammatory disease of large intestine. They all found it as a idiopathic chronic disease which causes inflammation in small and large intestine.

**1913:** A surgeon, Sir Kennedy Dalzeil came across 9 patients who are suffering from interstitial enterities. After proper diagnosis Dalzeil and Crohn conclude that the cause of this symptoms is not intestinal tuberculosis, these are due to some other disease [10].

**1914-1931:** Many researches are performed in this area to find the new disease by taking the base of Crohn's and Dalzeil's research.

**1932:** Crohn's Disease was discovered in this year by Burrell Bernard Crohn, Leon Ginzberg and Gordan D. Oppenheimer [11,12].

**1894:** Johne's disease was first reported in Germany by Dr. H A Johne and Dr. L Frothingham.

**1910:** Koch's postulates were fulfilled by Trowt by growing MAP in laboratory and transffering the disease to the experimentally infected cattles [13].

## Symptoms of Crohn's Disease

As the symptoms of disease varies according to the severity of disease but some common symptoms of crohn's disease are:

- Diarrhea
- Cramping and pain in abdomen
- Weight loss

Some other symptoms which may also seen during the infection of crohn's disease are:

- Fever
- Anemia
- Ulcers in digestive tract
- Joint pain
- Nausea or loss of apetite

## Complications of Crohn's Disease

Some complications which may occur during the crohn's disease are:

- Intestinal obstruction: It is also known as bowel's blockage as it blocks the movement of food and feacal material through the intestines. It may block the intestine fully or partially by thickening their walls so that the area for food and feacal transfer becomes narrow and the intestine becomes obstructed.
- Fistula: It refers to the abnormal passage between an organ or outside the body or it may be between two organs, so in CD the fistula is created through the walls of intestine and it causes inflammation.
- Abscesses: When there is inflammation through the walls of intestine it causes some more problems the patients body, the walls of intestine got swollen and pus formation is their which is refers to as abscessces.
- Ulcers: As there is inflammation through the walls of intestine, then this is also a possibility it may lead to ulcers in the digestive tract.

- Malnutrition: This condition arises when the body does not get the proper nutrients, vitamins, minerals which are needed by the body. As intestine will not function properly the body will not get the proper nutritions and condition leads to malnutrition [14,15,16].

## MAP

MAP is a member of M. avium complex. The strains of M. avium is universally present in the whole environment and also present in animals and human intestine. The M. avium strains normally do not cause any disease until the host is incapacitated or immuno repressed but on the other hand MAP specific pathogen can cause JD in many species of animals [17]. Besides this it has also been observed that MAP bacteria can live in animal tissue for many years and can cause any disease to animal. It will take around 2-10 minutes to show the symptoms which will also depend upon the level of exposure of bacteria and individual animals ability to fight against the infection [18]. MAP penetrates macrophages in lymphoid tissues in the ileum, there it will stops phagosomes maturation and activates the recruitment of inflammatory cells and it will cause granulomatus enteritis.
The characteristics which make MAP different from other mycobacterium species are: It is very sslow going bacteria, it is not able to produce mycobactin, it's possesion of the insertion elements IS900 that arise 14-18 copies in the MAP genome [19]. The 'gold standard' that will differentiate MAP from other mycobacterium species are the DNA sequence IS900 [20]. The entire IS900 gene is unique to MAP [21]. In contrast with other mycobacteria species, MAP is also differentiated by thick and waxy cell wall which contains 60% lipid due to which it shows the properties of acid fastness, hydrophobicity and also increase it's resistance towards chemical and physical processes [19]. The two main modes of transmission of MAP from host animals to humans are through the intake of contaminated water or milk [22]. The identification of IS900 insertion sequence found in MAP is done by PCR and also by in-situ hybridisation(ISH) and on this basis we can say that MAP is the cause of CD [23]. MAP spheroplasts (cell wall deficient forms) plays an important role for the cause of disease in humans and paucibacillary form of ID in other animal species.

## Detection of MAP in Crohn's Disease

MAP can be detected in CD patients but absent in control groups so it shows that relation of MAP in CD. The isolation of MAP by the means of culture methods is believed as a gold standards for detection of organisms whereas this is very time consuming process because the organism is slow growing in nature so molecular and serological methods are used as substitute [24].

## Routes of transmission of MAP

There are three routes of transmission of MAP to the human body:

- Water borne transmission: According to studies MAP infected organisms shed MAP in the environment (Rhodes *et al.,* 2014) and the organism will remain live on the agricultural slurry and for a long period of time and it will run off from their at either in vegetative state or in the form of spheroplast(McNees *et al.,* 2015). Pickup *et al.* (2005) reported that there were 32.3% of infection of MAP detected in the freshwater which is receiving runoff from the farming and domestic areas. There are some more studies on this area which reported the 80% MAP DNA contamination in the domestic water in Ohio USA (Sechi and Dow, 2015)
- Food borne transmission: The infected diary animals will shed the MAP infection in their milk also which is the main cause of food borne transmission of disease (Grant *et al*., 2015) whereas the another way of food borne transmission is the consumption of meat of diary animals (Gill *et al.,* 2011).
- Zoonotic transmission: Zoonotic transmission refers to the transfer of disease from animal to human. According to the publication of Dore *et al.* (2012) the infection will spread by direct contact of animal faeces and the main area of concern is that the infected animals remains asymptomatic during the subclinical phase which is very lengthy period of time(Naser *et al*., 2014).

## Detection of the Insertion Sequence IS900

PCR and ISH array are the main methods which are used to detect insertion sequence IS900. According to some earlier studies for the reliable and reproducible isolation and detection of MAP by using PCR is a difficult task as it needs the extraction of DNA from human tissue which is a difficult task and also sometimes false negative results are obtained because of suboptimal sample processing procedures [25].Recently,two meta aanalyses techniques have been reported which will detect MAP by using mucleic acid based techniques. It proves that the patients which are suffering from CD shows the presence of MAP in their gut, regardless whether CD patients are compared with people suffering from UC or not suffering from IBD [21,24]. But this remains inconclusive and have many disputes on it. PCR data also can't solve this dispute and left it as a question because it can only work on DNA which is isolated from live bacteria or from scattered debris of killer organism [26].

## MAP and genetic susceptibility to Crohn's Disease

There were a long-held theory which says that a genetically dysregulated host immune response to luminal bacteria can cause CD in host and there were two theories which came in support of it and it was positional cloning and Nucleotide Organization Domain 2/ Caspase Recruitment Domain 15 (NOD2/CARD15) are susceptible gene for causing CD [27]. The component which NOD2 receptor indentifies is muramyl dipeptides, which is a component of bacterial peptidoglycan, but the exact mecanism is still unknown. NOD2 polymorphism will lead to increasing propensity to develop CD. Due to deficiency of NOD2/CARD15 we observe abnormal growth and functions of Peyer's patches, also shows magnified immune response as well as increase the permeability [28]. That is why the patients having the mutations in NOD2/CARD15 gene are not able to control bacterial infections, shows

improper innate response so that the bacteria will grow and cause disease [29]. NOD2/CARD15 mutations cause the defective clearance of invasive Salmonella infections in epithelial cells and so as with MAP, they are responsible for the ineffective clearance of MAP. According to a study, the patients which have NOD2 mutated gene and are CD patients, their mononuclear cells have defective recognition of MAP bacteria [30].
In large population based studies done in Manitoba, have not observed any association between MAP serology nad NOD2 polymorphism [31]. But in small scale Sardinian study [32] says that there were high possibilities of association between MAP positive and NOD2 gene was mutated. Authours suggested that it might be a trend towards an association between the MAP positive and NOD2/CARD15 mutations. But when the CD subjects where compared against the controls which are CARD15/NOD2 mutated and the association is present in between them, but there were no association or impact is observed NOD2 mutated on MAP stauts among with CD patients [33].
According to genome wide study the novel susceptable gene for CD is interleukin-23 receptor (IL-23R) [34]. Evidences and researches shows that IL-23 and IL-12 genes are similar to each other and are responsible for the production of adaptive response which protect the body from intracellular pathogen like MAP [35]. Presently, there were two studies done which says that there is an association between CD and coding variant of autophagy related-16-like-1 (ATG16L1) gene [36] and IRGM gene [37], so they affect the innate immune response. This autophagy trafficking pathway is critical in inhibiting MAP bacteria in infected macrophages [38]. So, the infection can be caused by the mutation or defects in ATG16L1, IRGM, IL-23, NCF4 genes and many genes may not be reported yet [39].

***Aim***

To estimate bio-load and genotyping of MAP in human population in Agra region using IS900 PCR.

***Material Required***

- Eppendroff tubes
- Pipettes
- Autoclaved tips
- Water Bath
- Thermometer
- Deep Freezer
- Centrifuge machine
- Distilled water
- Triple distilled water

***Chemicals required***

- 5M NaCl
- 2M Tris Buffer
- 3M $MgCl_2$
- 0.4M EDTA
- SDS
- KCl
- $Na_2HPO_4$
- $KH_2PO_4$
- Tris HCl
- Ethanol
- Phenol
- Chloroform
- Isoamyl alcohol
- Proteinase-K
- Sodium Acetate

***Preparation of Buffer***

1. <u>RBC Lysis Buffer preparation:</u>
   5M NaCl - 440 microlitre
   2M Tris buffer - 1ml
   3M $MgCl_2$ $p^H$ 7.6 - 330 microlitre
   Distilled water - 200 microlitre

2. <u>WBC Lysis Buffer preparation:</u>
   5M NaCl - 2.04ml
   0.4M EDTA $p^H$ 8 - 5ml

2M Tris $p^H$ 7.6 - 1ml
10% SDS - 4ml
Distilled water - 200ml

3. 1X PBS ($p^H$ 7.6):
   NaCl - 8gm
   KCl - 0.2gm
   $NaH_2PO_4$ - 1.4 gm
   $KH_2PO_4$ - 0.24gm
   Triple distilled water - 1000ml
4. Ethanol (70%)
   Ethanol - 70ml
   Water - 30ml
5. Phenol:Chloroform:Isoamyl alcohol (25:24:1)
   Phenol - 25ml
   Chloroform - 24ml
   Isoamyl alcohol - 1ml
6. 3M Sodium Acetate
   Sodium acetate.$3H_2O$ - 40.8gm
   Water - 50ml

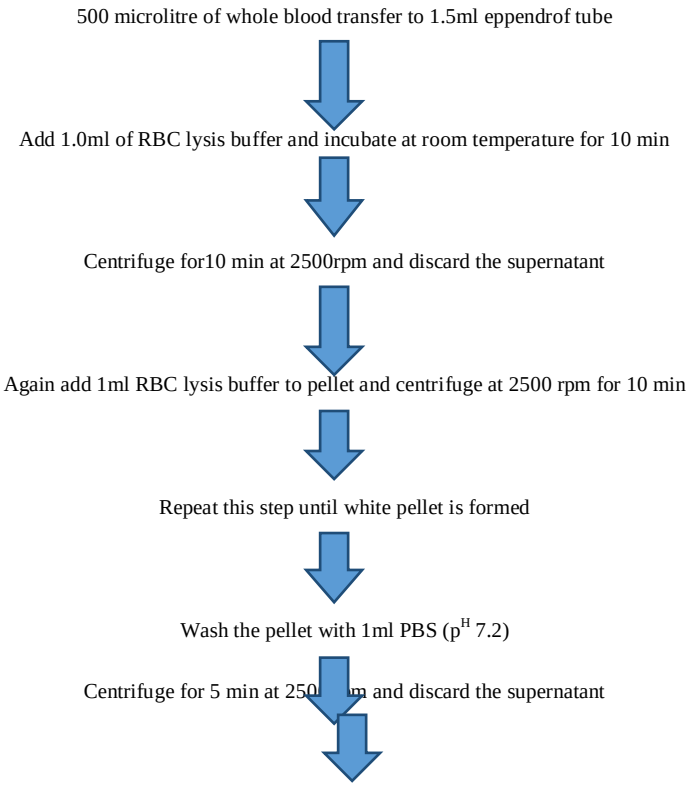
***Technology***

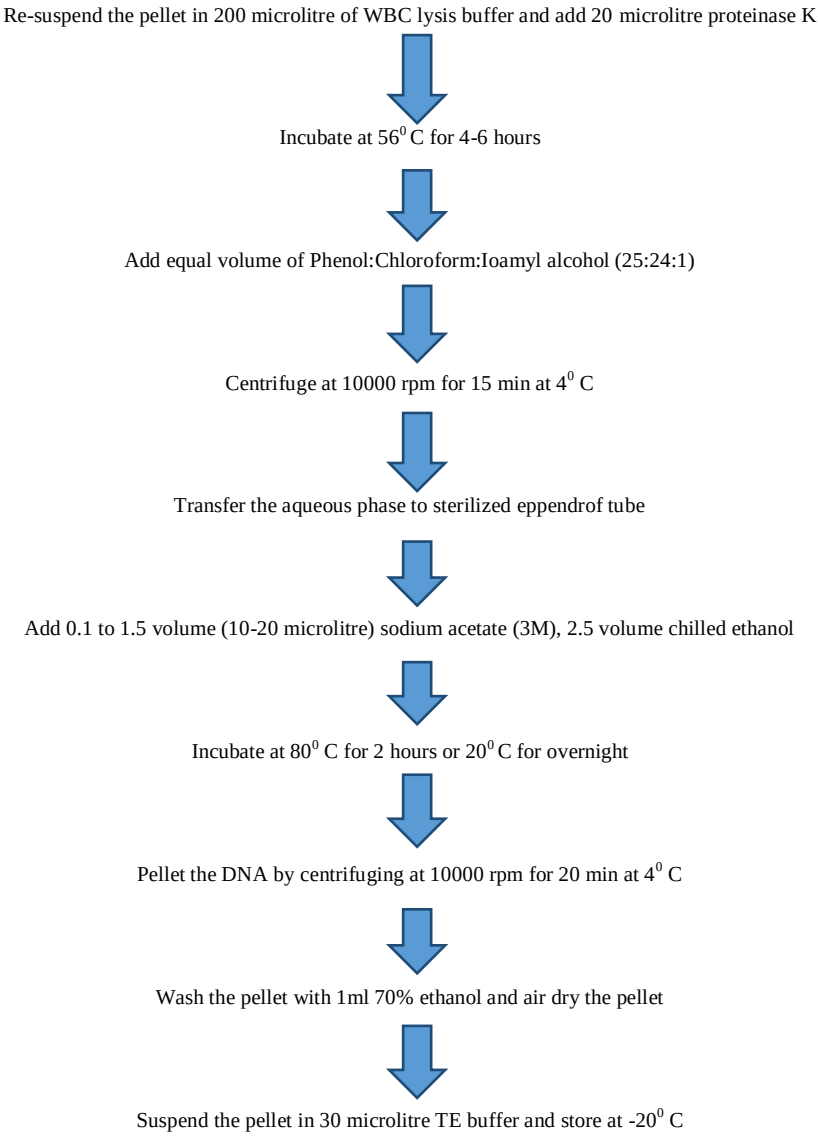
- Collection of blood samples
- Isolation of DNA from blood using RBC lysis buffer method
- PCR Amplification (IS900 blood PCR)
- Analysis of result

***Collection of blood samples***

Blood samples are collected from the people suffering from Type 1 diabeties. Informed consent from patients will be procured before taking blood samples from the patients.

***DNA Isolation from blood by RBC lysis buffer method***






***PCR Amplification (IS900PCR)***

PCR stands for Polymerase Chain Reaction. Its function is in-vitro DNA replication. It is based on Peltier effect. PCR is invented in 1983 by Dr. Kary Mullis. In PCR we will amplify a specific piece of DNA segment and can make millions of copies of that specific piece of DNA. PCR is completed in three main steps and all the steps have their own temperature:

1. Denaturation : 94-98$^0$ C
2. Annealing : 50-60$^0$ C (depends on Tm)
3. Extension : 72$^0$ C

Tm is Melting Temperature. Annealing should be done at the temperature less than 3-5$^0$ C by melting temperature
Tm = 4(G+C)+2(A+T)

***Components of PCR***

There are 5 main components of PCR:

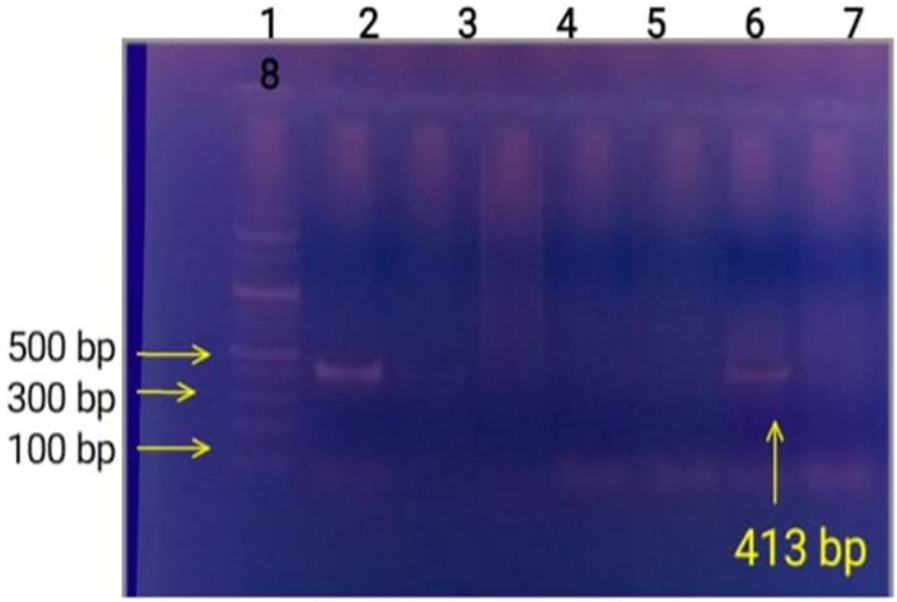
1. DNA template
2. Primers (Forward primer and reverse primer)
3. Taq polymerse
4. Deoxy nucleoside triphosphates (dNTPs)

5. Divalent cation ($Mg^{2+}$)

DNA Template: It is the piece of DNA which we have to amplify.The template DNA should be less than 3 kb and ideally it should be only 1 kb length.
Primer: The primers should be designed complimentary to the template DNA. The primers should be short and single stranded. The forward primer is also known as antisense strand and reverse primer is known as sense strand. The primer should be 18-28 bp length so that it will not bind to non target site or if it is longer than this then it will take more time for hybridization. It should have 40-60% GC content.
Taq polymerase: It is a DNA polymerase which is isolated from *Thermal aquaticus*. Its main function is to form the new strand from the complimentary strand by using dNTPs present in the master mix.
dNTPs: It contains dATP, dCTP, dGTP, dTTP. They all acts as building block for the new DNA strand.
Divalent cations ($Mg^{2+}$): It will act as a cofactor and help to increase enzyme activity. It helps in primer annealing (in proper binding of primer to its complementary sequence).

## Result

Study have reported moderate **'bio-load' of MAP (12.5%)** using IS*900* PCR in & around Agra region.The first lane is the DNA ladder which shows 100 base pair, 300 base pair, 500 base pair. The second lane is the positive control and 3-8 lane is isolaed DNA samples. MAP specific product is 413 base pair using IS900 specific primers confirmed the presence of MAP. Here, as the picture shows the sample 6 is positive to MAP.




## Conclusion

This pilot study provided the scenario of 'bio-contamination' of MAPin confirmed cases of diabetes in and around Agra region of UP, which will poses a serious threat to the public health. We have to spread awareness about the threat of this disease.

### References

[1] Ryan KJ; Ray CG, eds. (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill. ISBN 978-0-8385-8529-0.
[2] Neta Brender-Gotlieb, Merav Lidar, in Infection and Autoimmunity (Second Edition), 2015
[3] Mullan, W.M.A. (2015). HTST pasteurization. Is it time to raise statutory time / temperature conditions to destroy Mycobacterium avium subsp. paratuberculosis (MAP)?. [On-line]. Available from: https://www.dairyscience.info/index.php/food-model/277-htst-pasteurization.html . Accessed: 30 July, 2018. Updated October, 2017; April 2018.
[4] Cellier C, De Beenhouwer H, Berger A, Penna C, Carbonnel F, Parc R, et al. *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum* DNA cannot be detected by PCR in Crohn's disease tissue. *Gastroenterol Clin Biol.* 1998;22:675–8. [PubMed] [Google Scholar]
[5] Ellingson JLE, Cheville JC, Brees D, Miller JM, Cheville NF. Absence of *Mycobacterium avium* subspecies *paratuberculosis* components

from Crohn's disease intestinal biopsy tissues. *Clin Med Res*. 2003;1:217–26. [PMC free article] [PubMed] [Google Scholar]

[6] McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to M. para, as determined by DNA probes that distinguish between mycobacterial species. J Clin Microbiol 1987; 25: 796–801.

[7] Morgagni, B. The seats and causes of diseases investigated by anatomy in Five Books, containing a great variety of dissections and remarks. London: A. Miller, T. Cardelle and Jonston-Payne Publisher 1769, 2: 64. Egalement de Sedibus et causis morborum. Venise 1761.

[8] Combe, C. and Saunders, W. (1813) A singular case of stricture and thickening of the ileum. Medical Tranactions oj the College of Physicians (London) 4, 16-21

[9] Moore, N. (1882) Transactions of the Pathological Society of London, 34, 112.

[10] Dalzeil, T. K. (1913) Chronic interstitial enteritis. British Medical Journal, ii, 1068-70.

[11] Crohn, B. B. and Yarnis, H. (1966) Granulomatous colitis: an attempt at clarification. Journal of Mount Sinai Hospital New York, 33, 503-13.

[12] Ginzburg, L. (1986) Regional enteritis: historical perspective (B. Crohn and L. Ginzburg). Gastroenterology 90: (5 Pt 1), P: 1310-1, May 1986.

[13] Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant *paratuberculosis* Johne's disease: The current status and future prospects. *Cornell Vet*. 1984;74:218–62. [PubMed] [Google Scholar]

[14] Kappelman MD, Moore KR, Allen JK, Cook SF. Recent trends in the prevalence of Crohn's disease and ulcerative colitis in a commercially insured U.S. population. Digestive Diseases and Sciences. 2013;58:519–525.

[15] Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;142(1):46–54.

[16] Burisch J, Munkholm P. Inflammatory bowel disease epidemiology. Current Opinion in Gastroenterology. 2013;29(4):357–362.

[17] Uzoigwe JC, Khaitsa ML, Gibbs PS. Epidemiological evidence for Mycobacterium avium subspecies paratuberculosis as a cause of Crohn's disease. Epidemiol Infect. 2007;**135**:1057–1068. [PMC free article] [PubMed] [Google Scholar]

[18] Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J. 2006;**47**:874–882. [PMC free article] [PubMed] [Google Scholar]

[19] Rowe MT, Grant IR. Mycobacterium avium ssp. paratuberculosis and its potential survival tactics. Lett Appl Microbiol. 2006;**42**:305–311. [PubMed] [Google Scholar]

[20] Greenstein RJ. Is Crohn's disease caused by a mycoba-cterium? Comparisons with leprosy, tuberculosis, and Johne's disease. Lancet Infect Dis. 2003;**3**:507–514. [PubMed] [Google Scholar]

[21] Abubakar I, Myhill D, Aliyu SH, Hunter PR. Detection of Mycobacterium avium subspecies paratuberculosis from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. Inflamm Bowel Dis. 2008;**14**:401–410. [PubMed] [Google Scholar]

[22] Bannantine JP, Barletta RG, Stabel JR, Paustian ML, Kapur V. Application of the genome sequence to address concerns that Mycobacterium avium subspecies paratuberculosis might be a foodborne pathogen. Foodborne Pathog Dis. 2004;**1**:3–15. [PubMed] [Google Scholar]

[23] Chiodini RJ, Van Kruiningen HJ, Thayer WR, Coutu JA. Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. J Clin Microbiol. 1986;**24**:357–363. [PMC free article] [PubMed] [Google Scholar]

[24] Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M. Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. Lancet Infect Dis. 2007;**7**:607–613. [PubMed] [Google Scholar]

[25] Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J. Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J Clin Microbiol. 2003;**41**:2915–2923. [PMC free article] [PubMed] [Google Scholar]

[26] Chamberlin WM, Naser SA. Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses. Med Sci Monit. 2006;**12**:RA27–RA33. [PubMed] [Google Scholar]

[27] Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature. 2001;**411**:599–603. [PubMed] [Google Scholar]

[28] Barreau F, Meinzer U, Chareyre F, Berrebi D, Niwa-Kawakita M, Dussaillant M, Foligne B, Ollendorff V, Heyman M, Bonacorsi S, et al. CARD15/NOD2 is required for Peyer's patches homeostasis in mice. PLoS ONE. 2007;**2**:e523. [PMC free article] [PubMed] [Google Scholar]

[29] Barnich N, Darfeuille-Michaud A. Role of bacteria in the etiopathogenesis of inflammatory bowel disease. World J Gastroenterol. 2007;**13**:5571–5576. [PMC free article] [PubMed] [Google Scholar]

[30] Ferwerda G, Kullberg BJ, de Jong DJ, Girardin SE, Langenberg DM, van Crevel R, Ottenhoff TH, Van der Meer JW, Netea MG. Mycobacterium paratuberculosis is recognized by Toll-like receptors and NOD2. J Leukoc Biol. 2007;**82**:1011–1018. [PubMed] [Google Scholar]

[31] Bernstein CN, Wang MH, Sargent M, Brant SR, Collins MT. Testing the interaction between NOD-2 status and serological response to Mycobacterium paratuberculosis in cases of inflammatory bowel disease. J Clin Microbiol. 2007;**45**:968–971. [PMC free article] [PubMed] [Google Scholar]

[32] Sechi LA, Gazouli M, Ikonomopoulos J, Lukas JC, Scanu AM, Ahmed N, Fadda G, Zanetti S. Mycobacterium avium subsp. paratuberculosis, genetic susceptibility to Crohn's disease, and Sardinians: the way ahead. J Clin Microbiol. 2005;**43**:5275–5277. [PMC

free article] [PubMed] [Google Scholar]

[33] Sechi LA, Gazouli M, Sieswerda LE, Molicotti P, Ahmed N, Ikonomopoulos J, Scanu AM, Paccagnini D, Zanetti S. Relationship between Crohn's disease, infection with Mycobacterium avium subspecies paratuberculosis and SLC11A1 gene polymorphisms in Sardinian patients. World J Gastroenterol. 2006;**12**:7161–7164. [PMC free article] [PubMed] [Google Scholar]

[34] Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science. 2006;**314**:1461–1463. [PMC free article] [PubMed] [Google Scholar]

[35] Peyrin-Biroulet L, Neut C, Colombel JF. Antimycobacterial therapy in Crohn's disease: game over? Gastroenterology. 2007;**132**:2594–2598. [PubMed] [Google Scholar]

[36] Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet. 2007;**39**:207–211. [PubMed] [Google Scholar]

[37] Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, Roberts RG, Nimmo ER, Cummings FR, Soars D, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet. 2007;**39**:830–832. [PMC free article] [PubMed] [Google Scholar]

[38] Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004;**119**:753–766. [PubMed] [Google Scholar]

[39] Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008;**134**:577–594. [PubMed] [Google Scholar]