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Review Article

Mycobacterium avium subspecies *paratuberculosis* as a Foodborne Pathogen of High Public Health Significance

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ABSTRACT

Mycobacterium avium subspecies paratuberculosis (MAP) has potential zoonotic significance and is high risk infection for public health worldwide. It has broad host range and emerged as most successful pathogen of animals and human beings in last 3-4 decades. Animals infected with Johne's disease (JD) shed viable MAP in their milk, fecal, blood, and tissues. Transmission to human beings may occur directly via consumption of animal derived foods, through contact, handling and sharing of space with infected animals and indirectly through contaminated environment and consumption of milk and milk products made from pasteurized milk. Our study on 24,944 animals sampled in last 30 years has shown that MAP is endemic in the livestock population of the country (Cattle-43.5%, Buffaloes-28.6%, goats-21.5% and sheep-35.6%) using 4 diagnostic tests (microscopy, culture, ELISA and PCR). In another study after screening 27,703 human samples (24,322 serum, 3224 blood, 157 stool samples), we found that 34.1, 8.7 and 21.0% of human population was infected with MAP by indigenous serum ELISA, IS900 blood PCR and stool microscopy, respectively. In the absence of control programs bio-load of MAP is increasing with each passing day and if we take total population of animals (500 million plus) and human beings (1.23 billion plus) into account, infected population will go into millions. MAP is not inactivated during pasteurization therefore live bacilli are present in milk and milk products made from pasteurized milk. Presence of live MAP bacilli has also been reported from meat and meat products, soil, domestic water supplies, as well as river water. This article summarizes bio-incidences of MAP in environment, water supplies & aerosols, milk, meat and their products in India vis a vis global status. Besides discussing risk associated with presence of MAP in food chain and impact on human health, paper highlights the knowledge gap between Indian studies vis a vis International scenario.

Keywords: Paratuberculosis; Mycobacterium avium subsp. paratuberculosis; Food; public health; zoonotic; foodborne pathogen

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a major animal pathogen which has inflicted huge losses to the livestock industry worldwide (Vinodhkumar et al., 2013; Rawat et al., 2014; Bauman et al., 2016). It is very difficult to diagnose the MAP infection in the early stage of the infection. It has long been known and well established that animals infected with JD (clinical and sub-clinical), shed MAP bacilli in their milk and feces (Streeter et al. 1995; Raghuvanshi et al., 2013). Contamination in the milking parlor is one of the commonest source of contaminating milk with MAP (Nacy and Buckley, 2008). MAP is regarded as important food borne pathogen and has been reported to infect wide range of animal species, including non-human primates and human beings (Waddell et al., 2008; Kumar et al., 2010; Naser et al., 2009; KuKanich et al., 2013; Hussain et al., 2015). It may colonizes in animals for years without emerging clinical disease. Sub-clinically infected animals shed MAP in their milk (Shankar et al., 2010) and feces and contaminate pastures, environment and food chain for long time (Shankar et *al.*, 2010; Singh et al; 2012c). Live MAP bacilli has been also recovered from pasteurized milk (Ellingson et al., 2005), infant formulation made from pasteurized milk products (Hruska et al., 2011),

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surface water and soil samples (Singh et al., 2012c), cow manure ''lagoons'' that leach into surface water and municipal / tap water (Collins et al., 2003), thus providing MAP multiple routes of transmission to infect human population. Cow manure in solid and liquid forms is applied as fertilizer in agricultural land (Grewal et al., 2006; Gill et al., 2011), plants (upper greens, roots) and soil (surface and depth of 80 cm from plant roots) of agricultural and grazing areas hold MAP for longer period of time (Kaevska et al., 2014). MAP being thermo tolerant survives commercial pasteurization (72°C for 15-30 seconds) temperature (Grant et al., 1998) as well as high salt concentrations (Gao et al., 2002). Filtration, cold shock, hydrostatic pressure and pulsed electric fields procedures have already been tested on milk (Rowan et al. 2001). This superior survival efficiency and dormancy allows the pathogen to be more insidious in human beings (Whittington et al., 2005). Present paper reviews the public health significance of MAP infecting domestic livestock and related issues regarding presence in natural environment, survival abilities, pathogenicity and association with number of human diseases.

Association of MAP and Crohn's disease

JD is presently not in focus for the control and eradication either in the veterinary sciences field or in the medical sciences, therefore both animal and human population continue to get widely exposed to MAP infection from sub-clinical and clinical shedders of bacilli (Jones et al., 2006). MAP also has neuro-pathological and immune dys-regulatory properties (Scanu et al., 2007). This is why MAP is considered a strong candidate pathogen having association with CD (Momotani et al., 2012). Frequent isolation of MAP from CD patients and similarities of complication of CD matched with JD, which further confirmed the involvement of MAP infection with CD (Scanu et al., 2007).

Identification of MAP in CD patients has been accomplished by several different techniques. MAP has been cultured from the intestines (Sechi et al., 2005) and blood (Naser et al., 2004) of Crohn's disease patients. In addition, MAP has been identified in the intestines and blood of Crohn's patients by IS*900* PCR amplification (Naser et al., 2004; Abubakar et al., 2008). IS*900* PCR also detected MAP in two tissues of cervical lymph nodes of a 5 years child; later he developed CD (Hermon-Taylor et al., 1998). Multiple serological diagnostics have confirmed the antibody to MAP in human beings suffering with CD (Feller et al., 2007). CD share certain clinical as well as histo-pathological similarities with JD and is fast emerging as major disease of public health significance and a potential human zoonotic infection (Singh et al., 2012a). MAP has been detected in the tissues and blood of CD patients with a greater frequency (Naser et al., 2004), human breast milk of a patient with CD, positive antibodies to MAP antigens in blood samples of CD patients as compared to controls (Naser et al., 2000). In-situ DNA hybridization method has allowed direct visualization of small numbers of MAP in CD patient intestines by light microscopy (Abubakar et al., 2008). Initially, Dalziel had characterized the 13 cases of chronic intestinal enteritis where, he examined nine patients histologically and clinically and found that jejunum, transverse and sigmoid colon, as well as mid-ileum were affected (Dalziel, 1989) and patients had similar clinical findings as cattle suffering with JD. He therefore speculated that MAP could be a prospective etiological agent for the complications observed in human patients (Dalziel, 1989). Chiodini et al. (1984) was revived the association of MAP with CD and reported the isolation of un-characterized mycobacteria from tissues of three CD patients and Yoshimura et al. (1987) anticipated that pathogen present in a cell-wall deficient form was characterized as MAP. In a study, MAP was directly visualized by light microscopy in small numbers in the intestines of CD patients by ZN staining (Jeyanathan et al., 2007), while, other pathogen have also been detected in the intestines of patients with CD (De Hertogh et al., 2008). Some researchers argued that MAP has met both Koch's postulates (Greenstein et al., 2003) and Relman's criteria (Chamberlin et al., 2007) for microbial causation of CD. The causal association of MAP with CD remains controversial (Miller et al., 1996). Earlier studies from India on random screening of human population from from different geographical regions of North India showed 23.4 to 34.0% sero-prevalence of MAP (Singh et al., 2011; 2014b). Study reported moderately higher existence of anti-MAP antibodies in human population, which demands urgent measures and programs for reducing the bio-load of MAP in the animal population and in the environment as well.

MAP in water supplies and aerosols

Large number of studies reported survival of robust zoonotic pathogens in the environment (Szewzyk et

al., 2000). It has been suggested that there is a high risk of transmission of MAP to human population through drinking-water or by aerosols (Hermon-Taylor et al., 2000). Water bodies like lakes and rivers get contaminated by run-off of MAP from heavily grazed planktonic form of pastures, within protozoa or, more likely, both (Pedley et al., 2004). These organisms reaching domestic outlets in high dilution may aggregate in bio-films present in cold and hot household water storage tanks and delivery systems. Mycobacteria are well-known to present in aerosols and to concentrate in the water droplets (Blanchard & Syzdek,1972; Wendt et al. 1980).

Heavy rain falls from the Atlantic washes off contaminated pastures into rivers in spate. Taff (one of these rivers in spate), goes through the middle of Cardiff city. Research carried out during the 1970s in Cardiff, recorded significant increased incidence of CD ($p < 0.001$), but not of ulcerative colitis, in 11 of the local electoral city wards (Mayberry & Hitchens 1978). Of these high incidence wards, eight directly touched the river Taff and the three that did not, were closely adjacent to the North and East. This is the direction in which aerosols would be carried off by the usual South-Westerly winds (Hermon-Taylor 1993). Tracheal and bronchial inflammation with abnormal lung function tests were noticeable in the significant proportion of human population and chronic granulomatous tracheo-bronchitis in children with CD (Naser et al., 2009; Singh et al., 2012a). These are only few studies but much research is required on MAP presence and survival in the environment, in surface and ground waters and in the aerosols.

MAP in the natural environment

It is assumed that the survival of *Mycobacterium bovis* in the environment to be limited to hours or days. By contrast, MAP is physically more robust than other mycobacteria and is known to survive for months and perhaps years. Geographical regions having acidic soils enriched with humic and fulvic acids, boreal forests and areas with a heavy rainfall may favor the accumulation and persistence of MAP in the environment (Singh et al., 2012b; Hruska et al., 2012; Thomson et al.,2013; Rhodes et al., 2014). There are no reported studies to give us detailed understanding of the ecology and providence and presence of MAP in the atmosphere and potential cycling of MAP through human populations. It is very much possible that environmental MAP is taken up into protozoa and adapts in intracellular condition within protozoa present in the environment and in biofilm communities that profoundly influence microbial survival, phenotype and virulence. (Barker & Brown,1994; Hermon-Taylor et al., 2000). MAP grown in vacuoles in *Acanthamoeba castellanii* has developed the capacity of MAP to infect other amoebae, macrophages and human colonic epithelial cells including improved virulence for infection in a beige mouse model (Cirillo et al. 1997). MAP can remain alive for long periods inside the *Acanthamoeba polyphaga* (Steinert et al. 1998). The environmental exposure of MAP and its cycling through unicellular living beings as well as multi-cellular animal and human populations, has potential to have a profound effect on the evolution of these organisms and the evolution of strains with respect to adaptation and enhanced pathogenicity. Previous studies from England, France, Australia and US reported that soil types and soil composition have influence on maintenance, survival and repeated infection and incidence of JD in livestock herds (Dhand et al., 2009). Singh et al. (2012b) screened 71 samples from soil (51) river and water (20) (natural resources) of three districts of South Uttar Pradesh by microscopy and IS*900* PCR, 46.4 and 23.9% samples were found positive. They reported presence of MAP in holy river Yamuna, this may be due presence of large number of goshalas (cow shelters) for religious reasons in the holy city of Vrindavan (where samples were collected) and most of these cow shelters wash off 'cow dung' into the drains leading to river Yamuna, rather than lifting cow dung making compost. However, water samples from river Chambal with little human and animal population on its banks was comparatively free of MAP and other pathogenic micro-organisms. Similarly soil samples from farm area, where goatherds are reared over the years had soil samples positive MAP as compared to farmer's fields. besides heavy load of other microbial contaminants in soil, water and environment.

Risk associated with presence of MAP in human food chain

JD caused by MAP in animals is one of the most difficult diseases to diagnose and control because it has ability to resist adverse conditions in the natural environment. MAP is intracellular bacilli and JD infected animals, macrophages infected / laden with MAP can be found throughout the body of the animals. Most tissues including lymph nodes, spleen, bone

marrow, liver, kidneys, testis, fetuses, supra-mammary lymph node and lungs are affected (Kopecna et. al., 2008). It has been suggested that meat harvested from old dairy cows and used for making ground beef for human consumption may be a active source of MAP infection (Rossiter et al., 2001). Meat can also be contaminated with fecal material loaded with MAP bacilli during slaughter and procedures used for evisceration and processing of meat.

JD infected animals shed MAP bacilli in abundant quantities onto the land and pastures and increase the load of bacilli in the environment. Unlike *Mycobacterium bovis*, MAP is able to survive in the environment (Rowe et al., 2006).The contact time (CT) values for the effect of chlorine on MAP have been estimated to be up to 580-2300 times greater than those for *E. coli* (Wiley et al., 2012). Information regarding other zoonotic pathogens surviving in the environment suggests that there is a high risk that MAP may spread through aerosols and drinking-water supplies (Herman-Taylor et al., 2000). MAP is present in pasteurized milk (Slana et al., 2008), infant formulation made from pasteurized milk (Lund et al., 2002; Makharia and Singh, 2009), breast milk of a woman suffering from CD (2009), cow manure ''lagoons'' that can leach into surface ground water, cow manure (solid and liquid forms) applied as fertilizer to agricultural land, and municipal tap water (Whiley et al., 2012) thus providing multiple routes of transmission to human population. European Commission DG - Health and Consumer Protection in 2000 commissioned a report from the Scientific Committee on Animal Health as well as Animal Welfare on the possible links between Crohn's disease and MAP (EC, 2000). The National Association for Colitis and Crohn's disease (NACC) in UK commissioned a report from an expert review group into the evidence linking MAP and Crohn's disease in 2003 (NACC 2003). This report concluded that: 'both live and dead MAP' were present in human foods samples and the strongest evidence for this is in milk and DNA of MAP can be found in the bowel tissue of a proportion of patients with CD but also in lesser quantities in the bowel tissue of human beings having no lesions of CD. International Life Science Institute (ILSI), Europe in 2004 published a report from its Emerging Pathogen Task Force on MAP and the food chain (Gould et al, 2005). In its conclusion, it stated that "survival of MAP having public health concern depends on their possible involvement in human disease, in particular CD. At the present time, despite substantial research, the possible involvement of MAP in human diseases is still under discussion. This opinion followed a review of MAP survival characteristics in food which noted in particular, studies demonstrating survival of viable MAP in pasteurized milk. Food Standards Australia New Zealand conducted a microbiological review of the association between JD and CD (FSANZ, 2004). It concluded that CD may be a "multi-factoral disease or syndrome, with no etiological factor appearing to dominate. At present there is inadequate scientific evidence to confirm a conclusive link between JD (MAP infection) in ruminants and some cases of CD in human beings". The American Academy of Microbiology published a review on the evidence of pathogenicity in MAP (Nacy and Buckley, 2008). It has been noted that "there is a suspicion, supported by reports of genetic incompetence to interact correctly with certain bacteria and their products in some patients, that CD may have a currently unrecognized infectious origin, possibly environmentally derived". It has also been noted that "the option of more than one infectious reason that leads to a similar set of indications confounds the research agenda to find both a cause and a cure for CD". The report lists having five reasons why MAP has a suspected role in CD including MAP's ability to survive milk pasteurization and the success in some CD patients of antibiotic therapy against *Mycobacteria*. The report lays out the pros and cons of a possible association between MAP and CD. Finally, they concluded that "more research support and substantial additional research effort by both scientists and clinicians are necessary before we will know whether CD has an infectious aetiology and whether MAP is the offender".

There is lack of awareness in developing and under-developed countries on the presence and level of MAP infection in animals and human population (Singh et al., 2014a; 2014b). MAP has also been associated with various other human diseases / syndromes besides Crohn's disease in human population. Information on the association of MAP with different human health problems is yet to be recognized and taken seriously by the medical world globally.

JD or MAP infection is primarily a disease of food-producing domestic livestock (cattle, buffaloes, sheep and goats). Food products (meat and milk) derived from animals infected with MAP is commonly

contaminated much before harvesting, that is during the disseminative stage of JD, when pathogen exist in muscle meat, internal organs (intestines, mesenteric lymph nodes), colostrum and milk, and the un-born fetus (Brady et al., 2008). During harvesting of milk and meat from animals, there is second opportunity for the contamination of food products with MAP by contact with faeces of the animal infected with JD (Grant, 2010). Thus raw products obtained from MAP-infected animals are likely to be contaminated with MAP (Eltholth et al., 2009). Counts up to 560 MAP/ml of raw milk from individual cows have been reported (Grant, 2010). The question then becomes whether manufacturing processes or, cooking by the consumer will reliably kill MAP.

During food manufacturing processes, killing of MAP is based on pasteurization, specifically high temperature short-time (HTST) pasteurization (Grant et al., 2002b). Early laboratory studies were carried out with MAP inoculated milk in test tubes. In these studies, the milk was subjected to heat treatments similar to those used for pasteurization in holding tanks (holder pasteurizing) or for high-temperature short-time (HTST) pasteurization (Stabel et al., 2004). MAP was recovered from most of the samples of pasteurized milk. Studies with milk heated in apparatus (test tubes, capillary tubes, or laboratory pasteurizers) indicated that both holder and HTST pasteurization could be effective for killing (inactivating) of MAP in milk. In measurement of standard thermal tolerance parameters (STTP) for MAP in laboratory studies indicate that it is more heat-resistant than *Mycobacterium bovis and Coxiella burnetti* (milk-borne zoonotic pathogens) designed to be killed by pasteurization (Sung & Collins, 1998). Corroborating this are 3 independent retail milk surveys reporting recovery of viable MAP by culture from retail HTST pasteurized milk (Ellingson et al., 2005). Clearly, HTST pasteurization method kills large numbers of MAP bacilli in milk. However, evidences suggests that it does not kill 100% of MAP bacilli and post pasteurization MAP contamination is frequently occurring. This may be happening due to formation of clumps by the bacilli due to high lipid contents during initial heating phase. Within these clumps these bacilli survive the pasteurization temperature.

a. MAP in dairy products

Majority of MAP based infections occur through food producing domestic livestock. Milk derived from infected animals is generally contaminated before harvesting (milking). During harvesting of milk, there is a huge scope of contamination of milk and meat with fecal matter (Rademaker et al., 2007). Early studies were directed at investigating the presence of MAP in live and dead condition in raw and pasteurized milk using PCR technology (Ellingson et al., 2005). Irene Grant has extensively studied and published results in 2002: MAP presence was confirmed by culture in 1.6 and 1.8% of raw and pasteurized milk, respectively. Grant et al., (2002a) concluded that live MAP is infrequently present in commercially pasteurized milk in the UK. Ministry of Agriculture, fisheries and Food (MAFF), UK has started research that was continued by Food Standards agency, reported 1.7% prevalence of live MAP from pasteurized milk on retail sale (FSA, 2000) and pasteurized cow's milk was screened by IS*900* PCR. Field studies were done on retail pasteurized cow's milk for screening of MAP infection by IS*900* PCR in UK, though it was unable to distinguish between live and dead bacilli but study indicated high risk of transmission of MAP to human food chain (Miller et al. 1996). Queen's University Belfast screened PCR positive cow's milk samples with optimized decontamination protocols and immune-magnetic capture method where, 11.8% were positive, and by culture 1.8% of samples were positive for MAP (Grant et al. 2002a). In Switzerland, 19.7% of 1384 samples of bulk-tank milk were positive by IS*900* PCR again emphasized the risk of MAP in human food chain (Corti & Stephan 2002). Bulk-tank milk were positive by IS900 PCR.

MAP is not inactivated during pasteurization; therefore live MAP bacilli are present in milk and milk products made from pasteurized milk. Extensive use of milk products made from pasteurized milk globally, poses serious threat to human life. Initially this problem was restricted to most of the developed countries due to high dependence on pasteurized milk and milk products. But slowly this problem has started plaguing developing countries like India due to increasing use of milk products like ice-cream, milk coffee and condensed milk made from pasteurized milk. Since the use of milk products made from pasteurized milk increased dramatically in the country, therefore chances of contamination of human population with MAP has also increased. Bio-load of MAP has been estimated in different types of milk and milk products made from pasteurized milk of domestic

livestock species in different parts of world as well as in India.

b. MAP in meat products

MAP primarily affects the intestines and is found systemically in animal body with the advancing stages of JD (Rubery, 2002). In addition to the gastro-intestinal system, MAP has also been detected in milk, blood, semen, mammary glands, lymph nodes, reproductive organs and foetuses (Larsen et al., 1970; Sweeney et al., 1992b; Kopecna *et. al.,* 2008). MAP can be detected in these tissues of animals with sub-clinical infection. Therefore, it is possible that meat can be contaminated with MAP obtained from both clinically infected and apparently healthy animals. Meat could also be become contaminated with faecal material during slaughtering and processing procedures (Verma et al., 2014).

In a study MAP has been detected in the intestinal lymph nodes / faeces of 34.0% of healthy thin dairy cows and from 3.0% of healthy thin beef cows at slaughter (Rossiter & Henning 2001). MAP was also isolated from 3.0% in peripheral lymph nodes and 8.0% in liver of dairy cows and beef cows, respectively. Information is not available concerning the ability of MAP to survive during meat cooking and processing methods. It is likely that contaminated meat that is cooked at low temperatures or not processed thoroughly may contain live MAP. Though, the significance of meat as a potential vehicle of food-borne exposure to MAP relative to the potential exposure through milk is not known (Food Standards Australia New Zealand, 2004). It has been reported that fecal matter of slaughtered animals is the main source of MAP contamination in meat and meat product (Wells et al., 2009). Gwozdz et al (2000) has reported the evidence of MAP DNA in the blood and / or liver of many sheep with advanced symptoms of JD.

Time period wise bio-load of MAP in domestic livestock species in last 30 years (1985–2015): CIRG study

Of the 24,944 clinical samples (faecal, serum, blood) from goats, sheep, cattle and buffaloes screened by multiple diagnostic tests (fecal microscopy, fecal culture, serum ELISA and blood PCR) over the last 30 years (1985–2015), cumulative average bio-load of MAP was 25.5%. Livestock species-wise average bio-load in goats, sheep, cattle and buffaloes was 21.5, 35.6, 43.5 and 28.6%, respectively. Bio-load was significantly lower in goats ($P < 0.05$) than in sheep, buffaloes and cows. Average bio-load of MAP in 30 years (1985–2015) time period at five yearly intervals; 1985–90, 1991–95, 1996–2000, 2001–05, 2006–10 and 2011–15 was 11.4, 13.1, 11.1, 24.2, 28.9 and 42.8%, respectively and showed increasing trend. Time zone-wise, the average bio-load was 11.6, 27.0 and 38.8%, in the time period of 15 years (1985–2000), 10 years (2001–10) and 5 years (2011–15), respectively.

Time period-wise bio-load of MAP in human population in last 8 years (2008–2015): CIRG study

Bio-load of MAP was estimated in human population from different region of the country by 'mass screening'. Of the 24,322 serum samples screened by Indigenous ELISA, 34.1% populations were positive for MAP infection. Percent bio-load of MAP infection was 34.0, 49.2 and 31.7% in Period A (2008-13), Period B (2013-14) and Period C (2014-15), respectively by Indigenous ELISA. Of 3, 224 blood samples screened by PCR, 8.7% population was MAP positive. Time period-wise bio-load of MAP infection was 8.4, 18.1 and 17.4%, respectively by blood PCR. Screening of 157 stool samples by stool PCR, the bio-load was 21.0% and percent bio-load of MAP was 5.9, 55.5 and 44.7 in Period A, Period B and Period C, respectively. Time period wise cumulative bio-load was 30.2, 47.8, 30.5 in period A, Period B and Period C, respectively.

Conclusions

MAP is a very tough bacteria having strong zoonotic potential competent to infect wide range of hosts. It contaminates raw milk and meat derived from the infected animals and water runoff from farms contaminated with fecal matter of animals infected with MAP. Use of pasteurization and low heating conditions (roasting, smoking etc.) may help MAP to survive cooking and food manufacturing processes. There are lots of evidences suggesting, MAP is a potential candidate for causation of Crohn's disease. Meat, milk and other food products from animals infected with MAP serve as important means of transmission of MAP to human population through animal based food chain. The evidence are huge which

appear to indicate that MAP is proficient in surviving during food processing we applied to protect us from diseases, such as cooking and pasteurization. Clinical and pathological similarities of Crohn's disease with other mycobacterial diseases. Associated evidences suggest that presence of MAP in the environment and milk and meat supplies (animal-based food) are not safe and may help to spread MAP to human beings. Human infection can only be restricted, if domestic livestock population is cured of infection.

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Conflict of interests

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