Mol Biotechnol (2009) 42:30–40
DOI 10.1007/s12033-008-9137-5

REVIEW

# Oligonucleotide Microarray Technology and its Application to *Mycobacterium avium* subsp. *paratuberculosis* Research: A Review

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Published online: 6 January 2009


**Abstract** Microarrays represent a modern powerful technology, which have potential applications in many areas of biological research and provide new insights into the genomics and transcriptomics of living systems. The aim of this review is to describe the application of microarray technology for *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) research. The main focus points include a summary of results obtained for *MAP* using microarrays, examination of the fields of *MAP* research which are currently being investigated and possible areas of future research. This article is divided into two parts according to the type of nucleic acid used for array hybridisation. Articles related to *MAP* research using microarray technology are then divided according to the field of study, such as comparative genome analysis, diagnostics, expression or environmental studies.

**Keywords** *MAP* · Johne's disease · Crohn's disease · Potential zoonosis · Chip · Expression · Genomics

**Abbreviations**

| | |
|---|---|
| CD | Crohn's disease |
| cDNA | Complementary deoxyribonucleic acid |
| gDNA | Genomic deoxyribonucleic acid |
| GIs | Genomic islands |
| ELISA | Enzyme linked immunosorbent assay |
| IFN-$\gamma$ | Gamma interferon |
| IL-1$\alpha$ | Interleukin-1$\alpha$ |
| LSPs | Large sequence polymorphisms |
| *MAA* | *Mycobacterium avium* subsp. *avium* |
| *MAC* | *Mycobacterium avium* complex |
| MAC-T | Mammary epithelial cells |
| *MAH* | *Mycobacterium avium* subsp. *hominissuis* |
| *MAP* | *Mycobacterium avium* subsp. *paratuberculosis* |
| *MAS* | *Mycobacterium avium* subsp. *silvaticum* |
| MAPK pathways | Mitogen-activated protein kinase |
| MDBK | Madin–Darby bovine kidney epithelial cells |
| MHC class II | Major histocompatability class II |
| ORFs | Open reading frames |
| PBMCs | Peripheral blood mononuclear cells |
| PPD | Purified protein derivates |
| real-time RT-PCR | Real-time reverse transcription PCR |
| SAGE | Serial analysis of gene expression |
| TGF$\beta$ | Transforming growth factor-$\beta$ |
| TIMPs | Tissue inhibitors of matrix metalloproteinases |
| TRAF1 | Tumor necrosis factor receptor-associated protein 1 |

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## *Mycobacterium Avium* Subsp. *Paratuberculosis*

In general, mycobacteria are slender, non-spore-forming, rod-shaped and slow growing microorganisms. They belong to the family Mycobacteriaceae, whose characteristic feature is the presence of mycolic acids in the cell wall contributing to acid-fastness (often described as "acid-fast bacilli"). The distinguishing features of *Mycobacterium*

*avium* subsp. *paratuberculosis* (*MAP*) include a long generation time, tight clump formation in liquid media and mycobactin dependence. *MAP* was first described in Germany in 1895 by Professor Heinrich Albert Johne and his assistant Langdon Frothingham, and was identified as the causal agent of a ruminant inflammatory disease, called paratuberculosis or also known as Johne's disease [1]. It is described as chronic infectious enteritis affecting mainly domestic ruminants, such as cows, sheep and goats, but also in some cases other animal species including primates and humans. The disease is primarily located in the terminal ileum and colon and it is characterised by the depletion of health conditions, emaciation, decreased milk production and diarrhoea. Young animals are the most susceptible, nevertheless adult animals can be also infected, particularly in the case of higher doses of *MAP* or longer exposure [2–4].

Symptoms of paratuberculosis are similar to those of Crohn's disease (CD), which is a human systematic disorder manifesting itself in the chronic inflammation of the gastrointestinal tract. As in paratuberculosis, mainly the terminal ileum and colon are affected and the disease is usually characterised by abdominal pain, general malaise, loss of energy and weight, and other non-specific symptoms [5, 6]. A definitive explanation of CD causation does not yet exist, although *MAP* is thought to be one of the triggering factors [5–10]. Drinking water, water aerosol [11], milk [12–15] or even meat [16] are considered to be sources of *MAP* in human populations.

The complete genome of *MAP* (Strain K10) has already been sequenced and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). It comprises of a single circular chromosome of more than $4.8 \times 10^6$ base pairs, encoding 4,350 genes [17]. Compared to other mycobacteria (*M. tuberculosis*, *M. leprae* and *M. bovis*), the *MAP* genome is characterised by special characteristics which possibly play a role in its distinctive features. These include a higher number of regulatory genes, which may correlate with its ability to survive under diverse environmental conditions and increased redundancy in the genes connected with lipid metabolism, which probably result in a more robust cell wall structure and consequently the ability of *MAP* to colonise the intestine of ruminants [17, 18]. Although most of *MAP* genes have already been described, one-third of them remain to be functionally characterised. For more information about genomics of mycobacteria and *MAP* see reference [18].

## Microarrays

Microarray (array, microchip, biochip, DNA chip or gene chip) technology represents an important tool for the investigation of various areas of biology, mainly due its ability to simultaneously detect and analyse thousands of different genes in a single experiment. Miniaturisation, speed and accuracy are the other attractive characteristics of this approach. Traditional arrays contain nucleic acid probes immobilised on the solid surface of a chip. These hybridise with added nucleic acid molecules (targets), which are fluorescently labelled, commonly with Cy3 and Cy5 dyes. The fluorescence signal is subsequently scanned and the resultant image is processed by image analysis and data analysis software [19–23].

Microarray technology is increasingly being applied to many fields such as diagnostics, genetic and mutation analysis, evolutionary and expression studies, and environmental research. Today, various types of microarrays are utilised for the analysis of different target material, such as DNA [24–26], cDNA [27, 28], RNA [29], PCR products [30], proteins [31–33] and other materials allowing quantitative analysis. Common types of microarrays include oligonuceotide arrays, cDNA arrays, protein and tissue arrays [20]. For mycobacteria research, two types of microarrays are most commonly used: (i) high-density oligonucleotide arrays; and (ii) spotted microarrays.

High-density oligonucleotide arrays are produced by synthesising oligonucleotides directly on a chip by a photolithographic chemical reaction. This technique allows the production of a high-density chip containing thousands of short probes (15–70 nucleotides) targeted at different loci in a certain genome. Its salient feature is the presence of two types of probes—perfect match probe and mismatch probe; the latter differs from the former by a single base in the middle of its sequence. The chip contains several different sequences from the same gene, and therefore can be used to identify different variants (alleles) of the selected gene [34]. The Affymetrix GeneChip represents the current leading in situ hybridised chip [35, 36].

The use of the spotted array involves the independent synthesis of probes and their consequent spotting on a slide using a microarray spotter. In comparison with a high-density array, each probe of spotted array corresponds exclusively to a single gene or transcript. Various single-stranded or dissociated double-stranded nucleic acids can be used as probes, dependent on the type of microarray (gDNA, cDNA, synthetic oligonucleotides, PCR products or restriction-enzyme digested fragments). The length of the probe is commonly between 200 and 800 nucleotides, but in a case of synthetic oligonucleotides it could be much shorter i.e., from 50 to 70 nucleotides. This technique is now used through out the world to produce home-made arrays for the analysis of different types of samples. Moreover, it allows customisation when the species origin and number of probes should be chosen with regard to the studied material. For more information about spotted and

cDNA microarrays and their use, refer to the following reviews [37–39].

Whereas high-density oligonucleotide arrays are produced by commercial companies, spotted microarrays are commonly designed and prepared in research laboratories. However, to generate comparable and reproducible data, a spotted microarray platform has to fulfil various requirements. These include above all, appropriate experimental design, the presence of target molecules in sufficient quality and quantity, the design of probes, appropriate hybridisation conditions, the inclusion of a housekeeping gene in the case of expression studies, normalisation strategies or validation and data analysis. In particular, data mining and visualisation strategies are greatly important with regard to the huge amount of results generated by microarrays. For detailed information about the designing of microarray experiments and their evaluation see review articles [40–46].

## Application of Microarray Technology in *MAP*

Microarray technology is becoming more and more used by various types of research laboratories. Commercial chips containing the genomes of important or common bacteria are widely available. Unfortunately at present there is not a single commercial chip available for *MAP*. However, a few arrays containing the whole *MAP* genome have been developed [47–49]. Microarrays can be classified from many perspectives; in this review, they are divided according to the type of the target nucleic acid and subdivided according to their practical application (Table 1).

### Target Nucleic Acid: DNA

#### *Diagnostics*

The diagnosis of bacteria is based on commonly used techniques, such as microscopy, culture, biochemical tests or PCR diagnostics. Although these methods may be laborious, time consuming, expensive or not applicable in some cases, they still represent the "gold standard" for bacterial identification. Microarray analysis introduces a new direction in this field; nevertheless, their broad application is still limited by their higher price. However, in the case of clinical and food analysis, in which time is a critical factor, microarray technology can represent a suitable tool [50–52]. Another benefit of arrays is their

**Table 1** Type of arrays used in *MAP* research

| Type of array | Sources of samples | Target for array | Type of research | Citation |
|---|---|---|---|---|
| GeneChip (Affymetrix): unique 16SrRNA and *rpo* sequences | Clinical human isolates | *MAP* cRNA (from gDNA) | Diagnostics | [53] |
| DNA array with specific probes | Clinical human samples | *MAP* gDNA | | [54] |
| *MAP* whole genome array | Cattle, sheep | *MAP* gDNA | Comparative genome analysis | [47] |
| | Armadillo, bison, cat, cattle, goat, human, sheep, starling | | | [49] |
| | Cattle, goat, sheep, mink | | | [60] |
| *MAA* whole genome array | Cow, sheep | | | [59] |
| | Cow, sheep, goat, deer, human, pig, rabbit, unknown | | | [61] |
| | Cow, goat, human, deer, eland, oryx, white rhino | | | [62] |
| *MAP* and *MAA* whole genome array | Different labs (cattle strains) | | | [63] |
| Whole genome *MAP* array | *MAP* ATCC 19698 | *MAP* cDNA | Expression studies | [48] |
| Bovine cDNA microarray | *MAP* infected cows | cDNA from cow′s PBMC | | [87–89] |
| Bovine cDNA microarray | *MAP* infected cows | cDNA from ileal tissue | | [27] |
| Bovine cDNA microarray | *MAP* infected bovine macrophages | cDNA from macrophages | | [91, 93] |
| Human GeneChip (Affymetrix) | *MAP* infected bovine macrophages | cRNA (from cDNA) from macrophages | | [92] |
| *MAP* whole genome array | *MAP* infected bovine epithelial cells | cDNA from epithelial cells | | [96] |
| Bovine cDNA microarray | Leucocytes from *MAP* infected cows | cDNA from leucocytes | | [97] |
| *MAP* whole genome array | *MAP* (from cattle, sheep and human) infected human macrophages | cRNA (from cDNA) from macrophages | | [102] |

*Notes*: *cDNA* complementary DNA, *cRNA* complementary RNA, *gDNA* genomic DNA, *MAA Mycobacterium avium* subsp. *avium*, *MAP Mycobacterium avium* subsp. *paratuberculosis*, *PBMC* peripheral blood mononuclear cells

ability to distinguish between closely related pathogens (subspecies or isolates), identify virulence factors or reveal variations within a pathogen's genome. Such results can be then effectively used to identify drug resistance or find the best therapy [53–56]. The diagnostics of pathogens could be either surveyed using the species specific sequences or by expression patterns of certain gene sequences. While the first method is described in this section, the second one will be discussed in the chapter concerning RNA (cDNA) as an array target.

One of the first applications of a bacterial high-density DNA probe array was that developed for diagnostics of mycobacteria [53]. Discrimination among bacteria was based on 16S rDNA polymorphisms. While some minor discrepancies between phenotypic and genotypic identifications were observed, 26 out of 27 species were correctly identified. However, *MAP* and *M. avium* subsp. *avium* (*MAA*) were not identified individually because of having identical 16S regions on the array. According to the authors, this problem could be solved by lengthening the sequence region. Apart from the 16S rDNA region, the array also contained probes for the *rpoB* and *katG* region, which are responsible for rifampicin and isoniazid resistance, respectively [57, 58]. Using this array approach, single point mutations could be easily identified and thus clinical interpretation could be facilitated.

Tobler et al. [54] developed an identification method for mycobacteria based on the 65 kDa heat shock gene (also known as *hsp65* or *groEL2*). Their system comprised of a two-step procedure involving real-time PCR detection followed by subsequent microarray hybridisation. In total, 37 different mycobacterial species were tested, including *MAP*. For closely related species (with 95–100% sequence homology), like *MAP* and *MAA*, only one probe was designed. In such a case, the same hybridisation pattern was observed all the time for both species, and thus they could not be distinguished from one another.

The diagnosis of mycobacteria in human medicine is mostly focused on *M. tuberculosis* complex or on different non-tuberculous mycobacteria (e.g. *M. intracellulare*, *M. kansasii*, *M. gordonae* and *M. marinum*) which can cause opportunist infections, mainly in immuno-suppressed people [55, 56]. Moreover, the high level of similarity among individual members within *M. avium* complex (*MAC*), especially between *MAP* and *MAA* makes diagnosis more difficult. For the above reasons, a few scientific articles concerned with the diagnostics of *MAP* using array technology have been published [56].

### *Comparative Genome Analysis*

DNA microarrays represent a very powerful tool for comparative genomic studies. Their contribution in this area lies in the acquisition of useful information concerning subspecies or isolate discrimination, virulence factors identification, facilitating an understanding of host preferences and others, such as determination of disease markers. Hence, they can subsequently improve diagnostics or facilitate vaccine development. Although mycobacterial research is frequently focussed on the detection of genomic polymorphisms between *MAA* and *MAP*, other non-tuberculous species have also been investigated. DNA microarray technology helped to reveal large sequence polymorphisms (LSPs) which were either specifically presented in *MAA* and not in *MAP*, or vice versa [59–61]. In particular, two LSPs were revealed and their presence in various *MAA* and *MAP* isolates was confirmed as present or absent. Even though most of the identified LSPs lacked the sensitivity and specificity necessary for the diagnosis of *MAP* infection, the authors assumed that they could serve as infection or disease markers [61].

The finding of LSPs was confirmed by Wu et al. [62], who defined these areas as genomic islands (GIs). Using microarrays they found 24 and 18 GIs specific for *MAA* and *MAP*, respectively. They described two types of genomic rearrangements (i) inversions, possibly responsible for the regulation of significant antigen expression and (ii) insertions or deletions of GIs which could be important for pathogen survival in different environments. Interestingly, most Gl deletions were discovered in *MAP* isolates originating from wild life animals, suggesting that these animals can represent a potential source of genomic rearrangements in *MAP*. Moreover, an in-depth analysis of GIs among *MAA* and *MAP* isolates revealed a higher level of plasticity for *MAA* isolates as a result of their higher polymorphism rate, when compared with *MAP*.

Marsh et al. [47] focussed their attention on genetic variability among various isolates of one species; they compared *MAP* strains isolated from one sheep and two cattle isolates. Three large genomic deletions were found in the sheep isolate when compared to the cattle strains. Unfortunately, the functions of most of the missing genes are not known and therefore the effect of their absence or presence is unclear. Although the study was limited by using an array based only on a cattle strain genome, these results could help clarify differences and features of *MAP* strains originating from different hosts.

Afterwards, the deletions in sheep strains were confirmed by Paustian et al. [49], who identified the missing LSPs as the regions involved in various functions such as lipid and energy metabolism, virulence and transcriptional metabolism. Moreover, regions in sheep isolates, which were not present in cattle isolates, were discovered. These regions encode proteins involved in glycopeptidolipid biosynthesis, transcriptional regulation and virulence. According to the authors, they could contribute to

phenotypic differences between sheep and cattle isolates. *MAP* genomes from various isolates (cattle, birds, goats, bison and human) had a hybridisation profile similar to the reference *MAP* Strain K10 (a cattle genomotype). In any of the cattle genomotype isolates, no LSPs were found contrary to other studied mycobacterial isolates (*MAA*, *MAH* and *MAS*), which also served as references. This could imply either a wide distribution of cattle genomotype isolates of *MAP* in the environment or their higher capability of infecting various hosts [49].

The study of *MAP* genetic variability has revealed some important features, which may be important for understanding *MAP* behaviour, its divergence from other mycobacteria, or the production of certain proteins, which could be useful for diagnostic purposes. This approach was implemented on various *MAP* strains used to produce Johnin PPDs (purified protein derivates) in different laboratories [63]. It is known that large sequences presented in *MAA* are missing in *MAP* strains and vice versa [62]. In a study by Semret et al. [63], large genomic sequences responsible for Johnin PPD production were determined as missing in some *MAP* strains. According to authors, this absence could occur as a consequence of continuous in vitro passages, which could lead to the inability of the strain to synthesise the Johnin protein and/or to poor sensitivity of the Johnin reagents.

*Environmental Studies*

Research into microbial ecology uses several types of microarrays, mostly divided according to their applied targets [64]. Environmental arrays play an important role in the study of the relationships among bacteria from various environments [65], identification of new functional genes, metagenomic studies [66] or for the determination of genomic relatedness using comparative genomic analysis [56].

According to our information, no metagenomic or general environmental study concerning *MAP* has been carried out using microarray technology. A comparative approach using a whole-genome array was used by Marsh et al. [47] who compared *MAP* strains from sheep and cattle, by Wu et al. [62] who studied the genomic diversity of *MAP* isolated from humans, domesticated and wildlife animals, as well as by Semret et al. [63], who characterised *MAP* strains used for the production of PPDs. All these articles are described in detail in section 'Comparative Genome Analysis' of this review.

Target Nucleic Acid: RNA (cDNA)

Gene expression analysis is based on the assumption that cells react to changes in their environment by increasing or decreasing transcription of appropriate genes. These changes in expression levels can be detected or quantified by specific methods such as Northern blotting [67], S1 nuclease protection [68], sequencing of cDNA libraries [69] or serial analysis of gene expression—SAGE [70]. Today, these methods have been replaced by real-time reverse transcription-PCR (real-time RT-PCR) and microarray technology. Real-time RT-PCR represents an extremely sensitive, rapid and easy method for the precise determination and quantification of gene expression. Compared to real-time RT-PCR, microarrays represent a semi-quantitative rather than quantitative method, although their biggest advantage lies in reliable screening of huge amount of genes simultaneously in one experiment. Currently, real-time PCR and microarrays represent the most powerful techniques for expression profiling studies [71–74].

Most of the published *MAP* gene expression studies are concerned with paratuberculosis, although a few have been published for CD. These can be divided according to either the host response to *MAP* or directly according to the gene expression of *MAP* itself. To our knowledge, only one article relating to gene expression of *MAP* and its response to environment and stress factors has so far been published [48].

Wu et al. [48] subjected *MAP* to a temperature of 45°C, under acidic and oxidative stress to induce general stress and mimic the environment in abomasums or within macrophages, respectively. The array containing all of the *MAP*s predicted open reading frames (ORFs) which identified a set of genes with the same transcriptional pattern within all stress conditions. Most of the genes were induced under acidic pH, which could be connected with conditions in enteric macrophages and could highlight the importance of a change in pH for *MAP* pathogenesis. As expected by the authors, the experiments revealed the up-regulation of various sigma factors. Based on the data obtained, *sigH* was suggested to be involved in oxidative and heat stress, *sigD* and *sigG* in persistent infection, and *sigI* in adaptation to cold shock. These results are similar as those described for *M. tuberculosis*, where *sigH* was induced after heat and oxidative shock and *sigI* after mild cold shock [75, 76]. Not many studies have been carried out in this area until now; therefore additional research will be needed in order to understand the mechanisms of the *MAP* response to various conditions and its survival in such environments.

*Paratuberculosis—Gene Expression Analysis of Host Cells*

Paratuberculosis is commonly diagnosed by cultivation of faecal samples and light microscopy, a PCR method using IS*900* [77–79] and *F57* sequence [80] or by the detection of antibodies using the ELISA method [81]. In vitro measurement of cell-mediated immune responses based on

measurement of released gamma interferon (IFN-$\gamma$) offers the possibility to identify animals with sub-clinical paratuberculosis infection [82]. Compared to the above approaches, microarrays allow the detection of any phase of paratuberculosis, and moreover, they enable the study of gene expression profiles, which could provide information on the origin or behaviour of the disease.

All expression studies carried out on animal or human cell lines infected by *MAP* are based on the assumption that *MAP* infection alters the expression profile of host cells. For this reason different cell lines are exposed to mycobacteria and subsequently the level of their gene expression can be studied using microarrays. Due to the fact that macrophages play a fundamental role in the immune response to intracellular pathogens and *MAP* preferentially dwell within macrophages, many research groups use these types of cells for expression research (see review citation [83] for more information regarding macrophages and microarrays and citation [84–86] for macrophages and mycobacteria). Expression profiles of immune system host cells are mainly determined for cells infected by mycobacteria either in vitro or naturally.

Microarrays have been used frequently in the work of Coussens et al. [87–89] who employed them to observe the gene expression in peripheral blood mononuclear cells (PBMCs) after *MAP* infection. In 2002, Coussens et al. [87] found differences in gene expression profiles when comparing cows in clinical and sub-clinical phases of infection. Surprisingly, many genes were found to be repressed in cells from animals in the clinical phase, while activated in the case of animals in the sub-clinical phase. Although these results brought interesting informations, the authors themselves pointed out the low number of tested samples, which could limit the validity of their conclusions. Later they were able to confirm the differences in gene expression in host cells of infected and uninfected animals. They also showed that the common response to stimulation by *MAP* is generally manifested by gene down-regulation in host immune cells [88]. Coussens et al. [89] then affirmed their previous results concerning the different expression profiles in PBMCs infected by *MAP* and in control cells. Moreover, they showed an enhanced expression of transforming growth factor-$\beta$ (TGF-$\beta$) and tissue inhibitors of matrix metalloproteinases (TIMPs) whose enhanced expression could lead to tissue migration deficiency of the infected PBMCs. Finally, it was shown that *MAP*-infected PBMCs were highly pro-apoptotic as they were expressing genes involved in apoptosis at higher levels than control cells.

Aho et al. [27] also studied gene expression, but in contrast to previous articles they aimed their attention at tissues from paratuberculosis positive and negative cows. Two genes coding for tumour necrosis factor receptor-associated protein 1 (TRAF1) and interleukin-1$\alpha$ (IL-1$\alpha$), were found to be dramatically over-expressed in positive animals. Because TRAF1 is known to have an anti-apoptotic role, its increased expression could explain, according to the authors, macrophage accumulation in lesions associated with paratuberculosis. The over-expression of IL-1 and the subsequent toxicity of its protein could be connected with paratuberculosis symptoms.

It is known that bovine macrophages are able to kill *MAA* in vitro, but do not have the ability to kill *MAP* [90]. A comparison of macrophage expression response to *MAA* and *MAP* infection can therefore provide new information about the survival strategies of *MAP* and the defence mechanisms of host organism. Weiss et al. [91] tried to solve this problem using bovine microarrays and found out a decreased ability of *MAP*-infected macrophages to undergo acidification, although the expression of their vacuolar H+ ATPase was higher compared to those infected with *MAA*. As the major function of H+ ATPase is related to phagosome acidification, the authors assumed this could contribute to the inability of *MAP*-infected macrophages to be acidified. It was also demonstrated that some genes of *MAP*-infected macrophages were differentially expressed in different time intervals which could be connected with the inability of the macrophages to maintain the dynamic immune response against *MAP*.

Subsequently, Weiss et al. [92] used a human array to characterise the response of *MAP*-infected macrophages in comparison with unactivated macrophages and macrophages activated by incubation with *MAA*, IFN-$\gamma$ or lipopolysaccharide. Lower expression of major histocompatibility (MHC) antigen class II molecule in cells infected with *MAP* in comparison with unactivated cells, represented the most interesting observation. Low MHC II expression logically leads to the decreased capacity of affected macrophages to present antigens, and in the long term to a diminished immune response of the host organism. In general, many genes with various functions were differentially expressed, which gives evidence of complex interactions between macrophages and the attacking organism [92].

The work of Murphy et al. [93] had a similar focus to that of Weiss et al. [91, 92]. Similarly to Weiss et al., Murphy et al. [93] observed an increased expression of vacuolar H+ ATPase in *MAP*-infected macrophages. However, they observed similar expression profiles in most macrophages infected with *MAA* or *MAP*, even when some significant changes in expression were observed. Most of the differentially expressed genes belonged to or were somehow connected with three MAPK (mitogen-activated protein kinase) pathways (p38, ERK1/2 or JNK). Although MAPK pathways genes were rapidly activated in both *MAA*- and *MAP*-infected macrophages, the level of expression was considerably lower in the latter. The differences in the

MAPK pathway gene expressions could be related to the observed stronger induction of gene expression in macrophages infected with *MAA* than with *MAP*.

The presence of mycobacteria has been previously described in mammary glands and milk [94, 95]. Patel et al. [96] later demonstrated the ability of *MAP* to enter, survive and to persist within the bovine mammary epithelial cells (MAC-T) and subsequently their invasion into Madin–Darby bovine kidney epithelial cells (MDBK). *MAP* incubated in milk was more invasive for MDBK, when compared with *MAP* exposed to water or a broth environment. These results showed that an environment of high osmolarity could serve as a trigger for the expression of determinants connected with the *MAP* invasion of host cells. Patel et al. [96] used microarrays for obtaining detailed information about gene expression of *MAP* isolated from infected MAC-T cells. From the bulk of genes, 20 (mainly regulatory, metabolic or virulence connected genes) were strongly induced. Nevertheless, the precise functions of those genes are still unknown; further research is required in this field.

### *Paratuberculosis—Gene Expression Analysis of MAP*

Gene expression can not only provide useful information for the investigation of cellular responses to stress conditions or for the study of host immune system reactions to infection, but can also be used as a diagnostic tool.

The expression of genes can exhibit a specific expression signature, which may be unique for a particular disease caused by a pathogen. As paratuberculosis is characterised by a long sub-clinical phase, such signature identification could be very useful for the determination of the infection phase [97]. CD30L seems to be a potentially interesting candidate gene which can contribute to an expression signature specific for sub-clinical paratuberculosis. The use of microarrays and real-time RT-PCR identified CD30L and the gene encoding P-selectin to be differentially expressed in leucocytes originating from infected cattle when compared with controls. CD30L is a cytokine belonging to the tumour necrosis factor family regulating cell activation, proliferation and apoptosis. P-selectin, a membrane glycoprotein, serves as an adhesion receptor for leucocytes. Considering that P-selectin represents an important marker for other diseases and thus it is not specific for paratuberculosis, CD30L seems to be as a more suitable candidate [97].

### *Crohn's Disease—Gene Expression Analysis of Host Cells*

Microarrays are beginning to play an important role in clinical investigation directed at the discovery of biomarkers. They allow the analysis of thousands of biomarkers from relatively a few patients, compared to common models which require hundreds of patients for statistical validation of a single marker [98]. Whether or not *MAP* could constitute as a trigger for CD is yet to be confirmed. Although the isolation of living bacteria from human patients is very difficult, viable *MAPs* have been isolated from various tissues of Crohn's patients, including peripheral blood or human breast milk [6, 7, 9, 95]. However, the onset of disease as a result of an autoimmune response to *MAP* represents another possibility for CD etiology (for more information about *MAP* and CD see references [99–101]. The study of host–pathogen interactions and the response of host immune system can provide new evidence of *MAP* association with CD occurrence, and microarrays, in particular, can widely contribute to this.

In general, expression profiles of host immune cells infected by *MAP* originating either from paratuberculosis-infected animals or from Crohn′s patients, are mostly studied on animal cell lines (see section 'Paratuberculosis—Gene Expression Analysis of Host Cells'). Human cell lines are rarely used. To date, only Motiwala et al. [102] have used transformed human cells lines challenged by *MAP* infection and assessed the level of their gene expression using the *MAP* K10 microarray.

Motiwala et al. [102] examined transcriptional changes in the expression profiles of human monocytes after phagocytosis using *MAP* strains from CD patients, cattle and sheep. A shared global expression pattern of anti-inflammatory pathways for human and cattle isolates based on their anti-invasive entry into macrophages was shown. This was in contrast with sheep isolates, whose pro-inflammatory genes were strongly induced. Anti-inflammatory pathways in human and cattle isolates could be associated with an effective infection process and persistence, whereas pro-inflammatory processes in sheep isolates can indicate host adaptation, evolution and rarity in other hosts. The results of this study indicate the importance of the *MAP* genotype to host specificity.

## Conclusion

The most useful and employed applications of microarrays in *MAP* research are genomic and expression studies, although relatively few papers dealing with environmental or diagnostics aspects have been published. Genomic studies using microarray technology have revealed significant differences between *MAP* and *MAA* genomes, mainly manifested as gene deletions. These results have a great impact on the understanding of the pathogenic potential of *MAP*, its virulence and host specificity.

Expression studies have become increasingly popular mostly due to the invention of microarrays and their

implementation into practice. In *MAP* research, microarrays have been mainly used to ascertain the gene expression of infected host immune cells. Numerous studies have examined the up- and down- regulation of genes in *MAP* infected cells, in comparison with control (uninfected) cells or with cells infected with *MAA*. Research into the *MAP* response to stress conditions, and its survival in the environment, offers another field for the wide usage of microarrays. Expression studies can be utilised to elucidate the defence mechanisms of the host cells against *MAP* infection and determine *MAP* survival strategies allowing them to be used not only in veterinary, but also in human medicine. Although microarrays have already contributed significantly to paratuberculosis research, there are still many unresolved questions which need to be answered.

**Acknowledgements** The authors would like to thank Maria Vass (Veterinary Research Institute, Brno) for the critical reading and English corrections. This work was supported by the EC (PathogenCombat No. FOOD-CT-2005-007081) and the Ministry of Agriculture of the Czech Republic (Grants Nos. MZe0002716201 and QH81065).

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