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A Microarray-Based Analysis of Differentially Expressed Genes in Intracellular *Brucella abortus* 544 within Mdbk Cells

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Abstract

Background: Modulation of gene expression is a fundamental requirement for adaptation of intracellular *Brucella abortus*. Since most of the current understanding mainly focus on professional phagocytes and renal involvement is uncommon in brucellosis, our aim was to identify and analyze changes in *B. abortus* gene expression in response to intracellular environment within a bovine kidney cell line.

Methodology: *B. abortus* RNA were isolated from Madin-Darby bovine kidney (MDBK) epithelial cells during replicative phase and the transcriptional profile of intracellular B. abortus was characterized using microarray analysis.

Results and interpretation: The microarray analysis revealed a total of 1,623 differentially expressed genes of ≥ 2-fold–788 (25.44%, 788/3098) upregulated and 835 (26.95%,835/3098) down-regulated genes as compared with free-living *Brucella*. Among these identified genes, 81 and 185 were upregulated and down-regulated at ≥ 7-fold, respectively, showing a marked induction of genes involved in transcription and distinct repression of genes involved in translation, ribosomal structure and biosynthesis.

Conclusion: The identified genes in this study may provide new insights into the molecular interactions between *B. abortus* and non-phagocytic bovine cell line, MDBK. In addition, several differentially highly expressed transcripts were hypothetical genes with unknown function and/or unclassified which require further characterization due to their potential contribution in the virulence and strategy of Brucella to survive and proliferate within the host.

Keywords *Brucella abortus*; MDBK; Microarray; RNA

Introduction

Brucellosis, one of the most important zoonotic diseases worldwide, has managed to elude eradication and is readily transmissible to humans resulting to acute febrile illness and undulant fever that may progress to a more chronic form, virtually affecting all of the organs [1,2]. *Brucella* is classified in risk group III by the World Health Organization (WHO) laboratory biosafety manual and possesses important characteristics including no classic virulence factors such as exotoxins or endotoxins, its lipopolysaccharide (LPS) pathogenicity is not typical, and ability to invade and persist through inhibition of programmed cell death [2,3]. The progression of brucellosis into a chronic form is related to the pathogen's ability to persist for prolonged periods within host cells, evading the host's immune system throughout the infection [4,5]. After entering the host–most commonly in mucous membranes of the respiratory and digestive tracts, brucellae are taken up by local tissue lymphocytes, transmitted through regional lymph nodes into the circulation and subsequently seeded throughout the body with tropism for lymphoreticular and reproductive systems [3,4,6].

As a facultative intracellular pathogen, *B. abortus* is exposed to many different microenvironments during its lifecycle; hence regulation of gene expression is a fundamental requirement for its physiological adaptation [7]. Identification and analysis of these changes in Brucella gene expressions in response to intracellular environments for survival may hold the key to provide better understanding of its pathogenesis and bring new insights into the molecular interactions between *Brucella* and its host, particularly in non-phagocytic host cells. Among the techniques employed in identifying differences in global gene expressions is the microarray technology which provides a high-throughput screening method to simultaneously measure the expression levels of a large number of genes or to genotype multiple genomic regions, hence it is the most widely used method for profiling mRNA expression [8]. Microarray analysis was used to identify *B. abortus* genes necessary for its intracellular survival in RAW 264.7 cells and revealed that 7.82% (244/3334) and 5.4% (180/3334) of all *B. abortus* genes were up-regulated and down-regulated, respectively [8], and in our previous study, 25.12% (801/3190) and 16.16% (515/3190) of the total *B. abortus* genes were upregulated and down-regulated, respectively, at ≥ 2-fold within bone marrow-derived macrophages (BMDMs) [9].

Research has focused on identifying virulence factor-encoded genes involved in the replication and survival of *B. abortus* mainly within professional phagocytes; however, the interaction between this pathogen and epithelial cells is also crucial in the outcome of infection for a deeper understanding of the pathogenesis of brucellosis.

However, the current understanding on the molecular mechanisms and factors that *Brucella* employed within epithelial cells is limited [10]. A previous study investigated the relationship between the severity of apoptotic and autophagic cell death based on the distribution of *Brucella* antigens in the different tissues of aborted bovine fetuses due to natural infection and showed that the bacterial antigens were highly evident in the kidneys [11]. Interestingly, involvement of the renal parenchyma in the acute phase of brucellosis is very rare but generally manifested as acute interstitial nephritis, chronic interstitial nephritis or glomerulonephritis [12]. Although the involvement of kidney is not common, it has been reported that brucellosis is about ten times more prevalent in patients with renal failure and concluded that the disease can cause nephropathy [13]. Macrophages are the known primary target of *B. abortus* but has also been reported that the pathogen can invade a variety of other cell types including the Madin-Darby bovine kidney (MDBK) cells [14,15]. However, the pathogenic mechanisms of the pathogen within this cell line have not been elucidated at the cellular and molecular levels. To our knowledge, there have been no further studies done on the infection of *Brucella* in MDBK cells. Consequently, we focus on identifying critical genes involved in the interaction between *Brucella* and this epithelial host cell line MDBK cells.

Materials and Methods

Bacterial strain

The standard wild-type strains used were from *B. abortus* 544 (ATCC 23448) cultivated and maintained in *Brucella* broth or on agar (1.5%) (Becton Dickinson, USA). The bacterial culture used in the experiment was grown in broth at 37˚C with shaking until stationary phase was reached and serial dilutions on agar plates were performed in the assessment of the number of viable bacterial cells.

Cell culture

MDBK cell line (NBL-1) (ATCC CCL 22) was maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37˚C under 5% CO_2. All the reagents were purchased from Gibco (USA).

Bacterial infection assay

MDBK epithelial cells were cultured at a concentration of 1×10^6 cells per well in 6-well plate overnight. The medium was changed into fresh medium and then infected with *B. abortus* at multiplicities of infection (MOIs) of 100. The cells were centrifuged at 150 xg for 10 min and incubated at 37˚C under 5% CO_2 for 1 h. The cells were then washed with DMEM and incubated in fresh medium containing gentamicin (30 μg/ml) for 30 min to kill the remaining extracellular and/or adhered bacteria.

Brucella RNA extraction and purification

Isolation of intracellular *B. abortus* was done as previously described [9]. In brief, the infected cells were washed, lysed with distilled water and incubated at 37˚C for 5 min. The cells were then scraped and centrifuged at 14,000 xg for 2 min to collect the pellets. The pellets were resuspended in a 1 ml solution containing 890 μl distilled water, 100 μl RQ1 DNase Reaction Buffer (Promega, USA) and 10 μl RQ1 RNase-free DNase (1 μg/ml) (Promega, USA), and incubated at 37˚C for 30 min. The mixture was centrifuged at 8,000 xg for 2 min and the bacteria were pelleted at the bottom.

The bacterial pellets from free-living or intracellular *B. abortus* were incubated in 500 μl RNAse-free water at room temperature for 30 min. One ml of RNA Protect Bacteria Reagent (Qiagen, Germany) was added and then incubated further for 5 min. The mixture was centrifuged at 5,000 xg and the bacteria were dissolved in 1 ml solution containing 850 μl RNase-free water, 150 μl 1% or 10% sodium dodecyl sulfate (SDS) and 10 μl proteinase K (Promega, USA), and incubated at 37˚C for 1 h followed by the addition of 200 μl Chloroform (Sigma, USA). The total RNA extraction was performed using Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed using RNase-Free DNase set kit (Qiagen, Germany).

The integrity of RNA was assessed by standard denaturing agarose gel electrophoresis, and the quantity and quality of RNA was evaluated using Optizen POP Nano Bio spectrophotometer (Mecasys Co., Ltd, Korea).

Brucella RNA sequencing and analysis

Sequencing and analysis of bacterial RNA were performed as previously described [9]. In brief, libraries for Illumina sequencing were made using TruSeq Stranded mRNA Sample Preparation Kit (Illumina, USA) following the manufacturer's instructions. RNA sequencing was performed using HiSeq 2500 Sequencing System (Illumina, Korea) with single-end 50 bp sequencing. The sequence data for the reference genome retrieved from NCBI database was used to align with the quality-filtered reads using Bowtie 2. The genes were clustered into functionally related groups and metabolic pathway using eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases, respectively. Mapping results and differentially expressed genes were visualized and analyzed, respectively, using CLRNASeq™ Program (ChunLab, Korea). The genetic alterations of intracellular *Brucella* were determined by comparison with that of the gene transcription levels of free-living *Brucella*. The microarray analysis was performed using three biological replicates from each infection and control time point using different preparations of cells and the fold changes of genes were calculated from three different samples.

Results

Analysis of RNA quality

Intracellular *B. abortus* total RNA from *B. abortus*-infected MDBK epithelial cells were successfully isolated using the present protocol. RNA integrity was assessed both on agarose gel and spectrophotometry. Electrophoresis on a denaturing agarose gel showed distinct bands of 23S, 16S and 5S rRNA indicating that the total RNA of *B. abortus* was intact and spectrophotometric analysis revealed an OD260/OD280 ratio of >1.8 indicating that the RNA samples were of superior quality suitable for microarray analysis.

Determination of bacterial gene regulation during the course of infection

Brucella was demonstrated to achieve an intensive replication from 24-48 h post infection after reaching endoplasmic reticulum (ER)-like

compartment 14, hence the transcriptional profile of *Brucella* at the replicative phase (24 h post-infection) was investigated to better understand the pathogen's mechanisms of infection within a non-phagocytic bovine kidney cell line, MDBK. The microarray analysis of the bacterial transcripts with at least 2-fold changes revealed that the response of *Brucella* during its infection within host cells was repression of most of its genes. These differentially expressed transcripts were consisted of 788 (25.44%, 788/3098) upregulated and 835 (26.95%, 835/3098) down-regulated genes as compared with free-living *Brucella*. The products of most highly expressed and repressed genes were N-formylglutamate amidohydrolase (B977_RS107740) with a 77.56-fold increase and uncharacterized protein pYV0051 (B977_RS118555) with a 540.66-fold reduction, respectively. The replicative phase of Brucella within MDBK cells suggested down-regulation of most bacterial transcripts as the pathogen's strategy for its intracellular survival.

Functional analysis of bacterial transcripts

Among the 788 upregulated and 835 down-regulated genes analyzed by microarray assay, 81 upregulated and 185 down-regulated genes were differentially expressed by ≥ 7-fold. Functional analysis showed that these upregulated genes were mostly of unknown function followed by those that are involved in the transcription and in the transport and metabolism of amino acid, carbohydrate and inorganic ion (Figure 1 and Table 1).

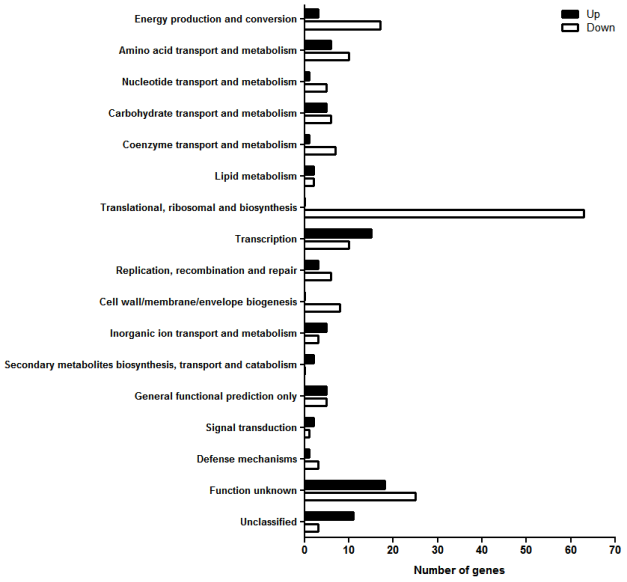

Figure 1: The differentially expressed transcripts of intracellular *B. abortus* at ≥ 7-fold within MDBK epithelial cells sorted by COG categories. Upregulated and downregulated genes of *B. abortus* at replicative phase were indicated as white and black bars, respectively. Data represent the average from at least three separated experiments.

Category	Accession No.	Protein	Fold
Energy production and conversion	B977_RS116170	Sulfite reductase	7.07
	B977_RS114775	Cytochrome c oxidase	10.24
	B977_RS118655	Aldehyde dehydrogenase family 2 member B4	15.1
Amino acid transport and metabolism	B977_RS106785	Pyruvate decarboxylase	7.79
	B977_RS105275	Spermidine/Putrescine ABC transporter substrate-binding protein	7.99
	B977_RS115580	NAD-dependent dihydropyrimidine dehydrogenase subunit PreT	8.19
	B977_RS107745	Urocanate hydratase	58.76
	B977_RS118750	usg family protein	72.86
	B977_RS107740	N-formylglutamate amidohydrolase	77.56
Nucleotide transport and metabolism	B977_RS107725	S-adenosylhomocysteine deaminase	23.13
Carbohydrate transport and metabolism	B977_RS106925	Putative binding protein BruAb2_0484	7.83
	B977_RS107770	Sugar ABC transporter substrate-binding protein	8.64
	B977_RS105020	ABC transporter permease	9.28
	B977_RS106780	5-dehydro-2-deoxygluconokinase	9.72
	B977_RS115915	Sugar ABC transporter substrate-binding protein	10.32
Coenzyme transport and metabolism	B977_RS116180	Siroheme synthase 1	8.61

Lipid metabolism	B977_RS110990	Isobutyryl-CoA dehydrogenase	7.01
	B977_RS116925	Methylcrotonoyl-CoA carboxylase beta chain	10
Transcription	B977_RS105675	TetR family transcriptional regulator	7.17
	B977_RS114710	UPF0301 protein BMEI1454	7.21
	B977_RS106620	Transcriptional regulator	7.38
	B977_RS114390	Transcription elongation factor GreA	7.48
	B977_RS107655	GntR family transcriptional regulator	7.55
	B977_RS106775	RpiR family transcriptional regulator	8.19
	B977_RS105895	GntR family transcriptional regulator	8.19
	B977_RS116080	Fis family transcriptional regulator	9.19
	B977_RS105765	MarR family transcriptional regulator	9.56
	B977_RS108350	GntR family transcriptional regulator	9.98
	B977_RS112035	Hypothetical protein	10.14
	B977_RS114380	AbrB family transcriptional regulator	10.8
	B977_RS107710	Aldehyde dehydrogenase	11.4
	B977_RS115215	Fur family transcriptional regulator	13.43
	B977_RS107720	GntR family transcriptional regulator	17.97
Replication, recombination and repair	B977_RS118540	Integrase	8.7
	B977_RS116150	Methylated-DNA--protein-cysteine methyltransferase	15.41
	B977_RS106070	Transposase	23.93
Posttranslational modification, protein turnover, chaperones	B977_RS113485	Arginyl-tRNA-protein transferase	11.03
Inorganic ion transport and metabolism	B977_RS116520	Sulfate-binding protein	7.63
	B977_RS108615	Iron ABC transporter substrate-binding protein	7.91
	B977_RS106815	Iron ABC transporter substrate-binding protein	7.99
	B977_RS116165	Probable phosphoadenosine phosphosulfate reductase	10.46
	B977_RS117800	ATPase	11.93
Secondary metabolites biosynthesis, transport and catabolism	B977_RS107730	Histidine ammonia-lyase	47.66
	B977_RS107735	Histidine ammonia-lyase	75.49
General functional prediction only (Typically, prediction of biochemical activity)	B977_RS105045	ABC transporter D family member 1	7.65
	B977_RS110255	Flagellar biosynthesis protein FlgM	7.85
	B977_RS111530	Protein DJ-1 homolog D	9.16
	B977_RS107210	ABC transporter substrate-binding protein	12.67
	B977_RS111260	Inosine-5-monophosphate dehydrogenase	32.33
Signal transduction	B977_RS112185	Universal stress protein	10.18
	ftrB	Transcriptional regulator	15.17
Defense mechanism	B977_RS119425	MFS transporter	7.54

Function unknown	B977_RS109985	Hypothetical protein	7.17
	B977_RS113610	Hypothetical protein	7.26
	B977_RS106970	Membrane protein	7.5
	B977_RS08290	Hypothetical protein	7.55
	B977_RS119015	Hypothetical protein	7.8
	B977_RS118615	Ribosomal RNA large subunit methyltransferase H	8.66
	B977_RS107110	Hypothetical protein	8.78
	B977_RS107905	Nodulation protein NodN	9.45
	B977_RS02240	Hypothetical protein	9.51
	B977_RS110395	Hypothetical protein	10.57
	B977_RS106975	Fusaric acid resistance protein	11.07
	B977_RS118610	Ribosomal silencing factor RsfS	11.61
	B977_RS112500	Hypothetical protein	11.83
	B977_RS112495	Hypothetical protein	12.38
	B977_RS118755	Fusaric acid resistance protein FusB	12.83
	B977_RS111555	Hypothetical protein	13.27
	B977_RS116250	Membrane protein	13.65
	B977_RS107750	Hypothetical protein	54.73
Unclassified	B977_RS109460	Hypothetical protein	7.22
	B977_RS0103625	Hypothetical protein	7.45
	B977_RS110120	DNA methyltransferase	7.55
	B977_RS106065	Transposase	7.86
	B977_RS115400	Hypothetical protein	8.06
	B977_RS110910	Hypothetical protein	8.15
	B977_RS0104155	Hypothetical protein	11.17
	B977_RS11365	Hypothetical protein	11.96
	B977_RS105565	Hypothetical protein	14.15
	B977_RS02335	Hypothetical protein	19.83
	B977_RS13980	Hypothetical protein	25.12

Table 1: *B. abortus* gene expression induced at 24 h post infection within MDBK cells.

Several of these genes were unclassified. On the other hand, the genes that appeared to repress were mostly involved in transcription, translation, energy production and conversion, and amino acid metabolism and transport (Figure 1 and Table 2). Similarly, several products of these highly repressed genes have unknown function and few were unclassified. Changed expression patterns of hypothetical genes were also observed which indicates that they may play important roles in the survival of *Brucella* within epithelial cells. Down-regulation of several ribosomal genes and RNA polymerase indicates amino acid starvation possibly due to poor nutritional intra-vacuolar microenvironment of *Brucella*. As a response, highly expressed genes that encode for transcriptional regulators are involved in regulation of virulence genes or bacterial responses to environmental stress. The products of the most highly expressed genes (B977_RS107745, B977_RS118750, B977_RS107735 and B977_RS107740) were involved in the metabolism and transport of amino acid suggesting the need for amino acids in the synthesis of proteins during the replicative phase; however, the products of the most highly repressed genes (B977_RS114825, B977_RS114830, rplT, B977_RS114840, B977_RS117320 and B977_RS113155) were involved in the translation,

ribosomal structure and biosynthesis indicating that the need for amino acids could be primarily as sources for carbon, energy and/or nitrogen. These findings indicate the regulation of *Brucella* genes as a mechanism for metabolic adaptation in response to changes in the nutritional environment for optimum utilization of the nutrients available in infected epithelial host cells.

Category	Accession No.	Protein	Fold
Energy production and conversion	B977_RS116810	Cytochrome o ubiquinol oxidase subunit III	8.35
	B977_RS113220	NADH:ubiquinone oxidoreductase subunit M	9.14
	B977_RS118220	Dihydrolipoamide succinyltransferase	9.22
	B977_RS118200	Malate dehydrogenase	9.44
	B977_RS105455	NADPH quinone oxidoreductase	10.61
	B977_RS118810	ATP synthase epsilon chain	11.14
	B977_RS113225	NADH:ubiquinone oxidoreductase subunit L	11.71
	B977_RS118210	Succinyl-CoA synthetase subunit alpha	11.8
	B977_RS118800	ATP synthase subunit gamma	16.57
	sucA	2-oxoglutarate dehydrogenase E1 component	16.94
	sucC	Succinyl-CoA ligase subunit beta	17.43
	B977_RS113060	Sorbitol dehydrogenase	18.35
	B977_RS111520	Isocitrate dehydrogenase	18.51
	B977_RS118795	ATP synthase subunit alpha	19.81
	B977_RS117910	Inorganic pyrophosphatase	20.3
	B977_RS118805	ATP synthase subunit beta	24.39
	B977_RS118790	ATP synthase subunit delta	30.56
Amino acid transport and metabolism	B977_RS105460	Leu/Ile/Val-binding protein homolog 8	7.08
	B977_RS110215	Aspartate aminotransferase A	7.34
	B977_RS109890	Anthranilate synthase	7.35
	B977_RS113990	4-hydroxy-tetrahydrodipicolinate synthase	7.35
	B977_RS116670	Argininosuccinate synthase	8.42
	B977_RS116890	3-phosphoshikimate 1-carboxyvinyltransferase	10.03
	B977_RS110705	Ketol-acid reductoisomerase	11.97
	B977_RS108680	Ornithine decarboxylase	13.81
	B977_RS115505	Acetylornithine aminotransferase	20.51
	B977_RS117485	Imidazole-4-carboxamide isomerase	7.93
Nucleotide transport and metabolism	B977_RS112190	Xanthine phosphoribosyltransferase	8.37
	B977_RS113075	Adenylosuccinate lyase	9.93
	B977_RS105375	Inosine-5'-monophosphate dehydrogenase	11.23
	nrdI	Protein NrdI	11.26
	ndk	Nucleoside diphosphate kinase	40.21
Carbohydrate transport and metabolism	B977_RS115180	MFS transporter	7.84

	gapA	Glyceraldehyde-3-phosphate dehydrogenase B	9.78
	B977_RS104685	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	11.95
	B977_RS108745	2-dehydro-3-deoxy-phosphogluconate aldolase	13.47
	B977_RS119075	Transketolase	13.8
	B977_RS119145	Inositol-1-monophosphatase	20.59
Coenzyme transport and metabolism	B977_RS116010	Putative thiamine biosynthesis oxidoreductase ThiO	7.06
	B977_RS116005	Phosphomethylpyrimidine kinase	7.28
	B977_RS116025	Thiamine-phosphate synthase	7.315
	B977_RS116015	Hypothetical protein	8.56
	B977_RS117135	S-adenosylmethionine synthase	8.77
	B977_RS113825	4-hydroxythreonine-4-phosphate dehydrogenase	9.19
	B977_RS117430	Adenosylhomocysteinase	18.23
Lipid metabolism	B977_RS105125	Hypothetical protein	7.12
	B977_RS114805	3-oxoacyl-ACP synthase	7.31
Translation, ribosomal structure and biosynthesis	B977_RS108305	Histidine--tRNA ligase	7.01
	B977_RS113850	Cysteine--tRNA ligase	7.11
	B977_RS110045	Peptidyl-tRNA hydrolase	7.32
	gatB	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	7.37
	B977_RS111420	30S ribosomal protein S14	7.62
	B977_RS112905	Serine--tRNA ligase	7.73
	B977_RS111465	30S ribosomal protein S13	7.88
	B977_RS116355	Tryptophan--tRNA ligase	7.89
	B977_RS111750	Glutamate--tRNA ligase 1	8.02
	B977_RS111425	30S ribosomal protein S8	8.11
	B977_RS112635	Valine--tRNA ligase	8.68
	rpmE	50S ribosomal protein L31	9.39
	B977_RS112425	Methionine--tRNA ligase	9.86
	B977_RS111430	50S ribosomal protein L6	10.15
	B977_RS111405	50S ribosomal protein L14	10.67
	B977_RS111395	50S ribosomal protein L29	11.1
	B977_RS110050	50S ribosomal protein L25	11.46
	B977_RS118565	50S ribosomal protein L21	11.47
	rpmH	50S ribosomal protein L34	11.83
	B977_RS111435	50S ribosomal protein L18	11.86
	B977_RS111410	50S ribosomal protein L24	13.71
	B977_RS118900	50S ribosomal protein L32	14.04

	B977_RS118570	50S ribosomal protein L27	14.08
	B977_RS111470	30S ribosomal protein S11	14.47
	B977_RS111440	30S ribosomal protein S5	14.51
	B977_RS111445	50S ribosomal protein L30	14.8
	B977_RS111450	50S ribosomal protein L15	14.84
	B977_RS117005	30S ribosomal protein S20	15.01
	B977_RS111415	50S ribosomal protein L5	15.52
	B977_RS117330	Translation initiation factor IF-3	15.88
	B977_RS111380	50S ribosomal protein L22	16.01
	B977_RS111400	30S ribosomal protein S17	16.62
	B977_RS106330	50S ribosomal protein L33	16.81
	B977_RS111685	Elongation factor Ts	17.1
	B977_RS104815	Ribonuclease P protein component	18.43
	B977_RS111390	50S ribosomal protein L16	18.86
	B977_RS118575	N-acetyltransferase GCN5	19.74
	B977_RS111385	30S ribosomal protein S3	21.26
	B977_RS111340	30S ribosomal protein S7	21.3
	B977_RS111375	30S ribosomal protein S19	21.65
	B977_RS111310	50S ribosomal protein L10	21.93
	B977_RS111360	50S ribosomal protein L4	22.05
	B977_RS117815	50S ribosomal protein L28	22.54
	rplB	50S ribosomal protein L2	23.31
	B977_RS111365	50S ribosomal protein L23	23.86
	B977_RS111355	50S ribosomal protein L3	24.36
	B977_RS111335	30S ribosomal protein S12	24.77
	B977_RS118690	30S ribosomal protein S16	24.95
	rpsJ	30S ribosomal protein S10	25.37
	B977_RS119150	Elongation factor P	25.49
	B977_RS111480	50S ribosomal protein L17	26.7
	B977_RS111315	50S ribosomal protein L7/L12	27.93
	tuf	Elongation factor Tu 1	28.07
	fusA	Elongation factor G	28.2
	B977_RS106125	Ribosomal protein S12 methylthiotransferase	28.59
	B977_RS117090	Polyribonucleotide nucleotidyltransferase	34.5
	B977_RS112765	Peptide chain release factor 2	39.36
	B977_RS113155	30S ribosomal protein S4	43.72

	B977_RS117320	50S ribosomal protein L35	44.78
	B977_RS114840	50S ribosomal protein L9	53.78
	rplT	50S ribosomal protein L20	64.9
	B977_RS114830	30S ribosomal protein S18	103.4
	B977_RS114825	30S ribosomal protein S6	206.57
Transcription	B977_RS111325	DNA-dependent RNA polymerase subunit beta	8.5
	B977_RS111320	DNA-directed RNA polymerase subunit beta	8.97
	B977_RS118580	N-acetyltransferase GCN5	11.86
	B977_RS117585	Transcription termination factor Rho	13.54
	B977_RS110175	Transcription elongation factor GreA	14.46
	B977_RS110225	Cold shock protein CspA	14.84
	B977_RS119120	Probable transcriptional regulatory protein BQ11790	15.22
	B977_RS105450	HTH-type transcriptional regulator McbR	17
	B977_RS111475	DNA-directed RNA polymerase subunit alpha	24.74
	B977_RS110400	ArsR family transcriptional regulator	7.78
Replication, recombination and repair	B977_RS106085	Transposase	13.56
	B977_RS112160	ATP-dependent RNA helicase-like protein DB10	32.45
	B977_RS116440	DEAD-box ATP-dependent RNA helicase CshB	33.82
	B977_RS119480	Transposase	41.19
	B977_RS109550	Transposase	41.19
	B977_RS118555	Integrase	540.69
Cell wall/membrane/envelope biogenesis	B977_RS112080	D-alanyl-D-alanine carboxypeptidase DacF	7.51
	B977_RS115700	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	9.31
	B977_RS113065	Lauroyl acyltransferase	12.22
	B977_RS115385	Antiholin-like protein LrgB	16.34
	B977_RS110180	Lipopolysaccharide core biosynthesis mannosyltransferase LpcC	19.18
	B977_RS117240	Hypothetical protein	110.41
	B977_RS110440	Lytic transglycosylase	209.1
	B977_RS107825	Hypothetical protein	76.47
Posttranslational modification, protein turnover, chaperones	B977_RS105230	Glutaredoxin	8.56
	B977_RS108265	Molecular chaperone GroES	11.06
	groEL	Molecular chaperone GroEL	11.23
	B977_RS118135	Peptidylprolyl isomerase	12.87
	B977_RS117325	Isoprenylcysteine carboxyl methyltransferase	19.18
	B977_RS111980	Peptidyl-prolyl cis-trans isomerase	10.44
Inorganic ion transport and metabolism	B977_RS116030	ABC transporter ATP-binding protein	8.6

	B977_RS113140	Bcr/CflA family drug resistance efflux transporter	8.62
	B977_RS107495	Magnesium transporter MgtE	29.63
General functional prediction only (Typically, prediction of biochemical activity)	B977_RS106165	Invasion protein B	7.87
	B977_RS117130	tRNA (guanine-N(7)-)-methyltransferase	8.11
	B977_RS119285	Glutamine amidotransferase	10.19
	B977_RS111585	NADPH-dependent 7-cyano-7-deazaguanine reductase	12.44
	B977_RS115390	Murein hydrolase transporter LrgA	20.08
Signal transduction	B977_RS107490	GTP-binding protein TypA	14.79
Intracellular trafficking and secretion	B977_RS104810	Membrane protein insertase YidC	7.35
	B977_RS111795	Preprotein translocase subunit SecG	10.69
	B977_RS118130	Protein translocase subunit SecA	10.92
Defense mechanisms	B977_RS116055	Hypothetical protein	25.6
	B977_RS116050	ABC transporter	28.18
	B977_RS116060	ABC transporter	35.29
Function unknown	B977_RS117915	Uncharacterized protein Atu26591	7.57
	B977_RS108245	Hypothetical protein	7.77
	B977_RS113300	Hypothetical protein	8.13
	B977_RS106190	Hypothetical protein	8.16
	B977_RS110265	Aspartyl-tRNA amidotransferase subunit B	8.26
	B977_RS115170	Polysaccharide deacetylase	8.3
	B977_RS119280	Membrane protein	9.18
	B977_RS119040	Hypothetical protein	9.28
	B977_RS118225	Membrane protein	9.52
	B977_RS107895	Hypothetical protein	9.57
	B977_RS110130	Hypothetical protein	9.67
	B977_RS109645	Hypothetical protein	9.88
	B977_RS110160	Membrane protein	10.04
	B977_RS112355	Hypothetical protein	10.32
	B977_RS114835	Hypothetical protein	10.35
	B977_RS113120	Hypothetical protein	10.87
	B977_RS114075	AP endonuclease	10.98
	B977_RS119390	Hypothetical protein	11.05
	B977_RS118480	Hypothetical protein	11.26
	B977_RS119290	Capsular polysaccharide biosynthesis protein J	12.98
	B977_RS117125	Ribosome maturation factor RimP	16.24
	B977_RS116645	Hypothetical protein	17.34

	B977_RS107330	Hypothetical protein	21.32
	B977_RS117310	Serine/threonine protein kinase	34.75
	B977_RS111680	30S ribosomal protein S2	9.04
Unclassified	B977_RS111105	Hypothetical protein	9.11
	B977_RS01000000119490	Hypothetical protein	16.27
	B977_RS116650	Hypothetical protein	23.58

Table 2: *B. abortus* gene expression repressed at 24 h post infection within MDBK cells.

Discussion

Intracellular bacterial pathogens are equipped with specific virulence genes regulated at the transcriptional level that aid in their ability to survive and replicate within host cells [17]. The pathogenicity of *Brucella* is due to its ability to adapt to its environmental conditions encountered, hence understanding the bacterial response and the molecular characterization of the pathogen's intracellular survival process would provide guidance for subsequent development of new therapeutic agents against brucellosis since infectious diseases are a major health threat representing the main cause of mortality worldwide [17,18]. However, most of the published studies primarily described the host response to infection. Consequently, a powerful and promising approach to identify the bacterial response at the transcriptional level is through microarray techniques.

Bacterial regulation of gene expression is a fundamental requirement for physiological adaptation; hence, the response of *Brucella* in its intracellular replicative niche at 24 h post infection at the transcriptional level was investigated and showed that the pathogen transcriptional profile with at least 2-fold changes was slightly downregulated. These findings were different from the response of *B. abortus* within professional phagocytes–both in RAW 264.7 cells and a primary cell line BMDM cells where most of the differentially expressed genes were upregulated revealing a unique mechanism of this pathogen to survive and proliferate in a different non-phagocytic host cell line. Furthermore, the present study showed higher rate of both upregulated and downregulated genes as compared to previous studies on phagocytes. Interestingly, few of these downregulated genes were previously reported as potential vaccine candidate against brucellosis which include nucleoside diphosphate kinase (Ndk), 50s ribosomal protein L7/L12 (RpIL) and phosphoglycerate kinase (Pgk) [19-21]. Many of these differentially regulated genes with ≥ 7-fold change are known to respond to stress such as amino acid starvation as a consequence of poor nutritional intra-vacuolar microenvironment of the pathogen. The most highly expressed genes found in the present study were associated with the transport and metabolism of amino acid suggesting the need for amino acids during the replicative phase of Brucella within MDBK epithelia cells. A wide range of microbial species utilize histidine as both a carbon and nitrogen source, and the N-formylglutamate amidohydrolase is known to catalyze the terminal reaction in the five-step pathway for histidine utilization in Pseudomonas putida [22]. The usg family protein may function as a subunit or stabilizer of phosphoribosylanthranilate isomerase (trpF) that catalyzes the fourth step of tryptophan biosynthesis which is important precursor to a large number of complex microbial natural products [23,24]. Polyamines, particularly spermidine and putrescine, have been associated with bacterial virulence and pathogenicity in human pathogens and in *B. ovis*, the defective uptake of spermidine and putrescine was contributed to the possible lack of bacterial virulence in humans [25]. Taken together, this suggests that *Brucella* is actively using the host amino acid for virulence and survival possibly as sources of carbon and/or nitrogen.

Brucella has been found to be able to survive and replicate within membrane-bound compartments of non-professional phagocytes, and few *Brucella* genes have already been identified such as the type IV secretion system (T4SS), which is encoded by the virB operon that has been found to be involved in the maturation process of *Brucella*-containing vacuoles (BCVs) constituting a major determinant of *Brucella* virulence in HeLa cells [26]. Similarly, we observed upregulation of *virB* genes (*virB1* to *virB11*; data not shown) although virB1 to *virB7* were observed to increase at ≥ 2-fold indicating that they play an important role in the intracellular survival of the pathogen in MDBK epithelial cells. The expression of the T4SS-encoding *virB* operon is regulated by multiple transcription factors belonging to different families including a MarR-Type regulator [27] which is similar to our study where a high expression of MarR family transcriptional regulator (10.8-fold) was observed. Bacterial survival in environment exposed to variations in nutrient availability and presence of toxic molecules requires a wide range of rapid, adaptive responses triggered by regulatory proteins [28]. Many of the upregulated genes in this study were involved in the transcription. Particularly, the transcriptional regulators belonging to MarR family of transcription factors aid in the regulation of bacterial responses to environmental stress [27]. Several transcriptional regulators belonging to TetR, GntR, RpiR, Fis, AbrB, Fur families were observed to increase at ≥ 7-fold. Proteins of the TetR family are involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, response to osmotic stress and toxic chemicals, control of catabolic pathways, differentiation processes and pathogenicity [28,29]. The transcriptional regulator gene, gntR, has been shown to play an important role in the control of *B. melitensis* virulence, and the GntR regulators to play important roles in maintenance of fatty acids concentrations, amino acid catabolism, organic acids, regulation of carbon catabolism and degradation of complex organics, as well as reported to affect expression of T4SS and quorum sensing system (QSS) in Brucella during infection in macrophages [29]. Attenuated six mutants that belong to the GntR family were identified and confirmed to replicate at lower rates in murine BALB/c model, while only one was slightly attenuated in HeLa cells indicating their role in *Brucella* virulence and proposed that GntR4 was a direct or indirect activator of *virB* transcription [30]. A conserved transcriptional regulator Fis was reported to be involved in the virulence of *Dickeya zeae* which is a causal agent of rice foot rot

disease [31]. Binding of metal ions to DNA is typically required by Fur family proteins to exert their transcriptional regulatory activities such as utilizing iron as a co-repressor and represses siderophore synthesis in pathogens that directly or indirectly controls expression of enzymes that protect against toxic reactive oxygen species (ROS) damage [32,33].

The interaction between the pathogen and host includes uptake and secretion of substances facilitated by a family of transporters. The ATP-binding cassette (ABC) transporters use a variety of substrates such as amino acids, sugars, inorganic ions, polysaccharides, peptides and proteins [34] and several ABC transporters have been associated to the full virulence and survival of *Brucella in vitro* and *in vivo*, hence the increase in the transcription of genes that encode for these proteins could help in the virulence and survival of *Brucella*. MFS transporter was also observed to increase which functions as a secondary carrier that transports small solutes in response to chemiosmotic ion gradients and its upregulation may be important for the adaptation of *Brucella* in the ionic intracellular environment [12]. Furthermore, several transcripts for hypothetical protein were observed to increase suggesting their important roles in the survival of *B. abortus* in epithelial host cells, thus impose special attention to be studied further.

Conclusion

In summary, the present microarray analysis identified 81 and 185 genes that were upregulated and down-regulated at ≥ 7-fold, respectively in *B. abortus* within MDBK epithelial cells at 24 post-infection as compared with free-living *Brucella*, showing a marked induction of genes with unknown function followed by those involved in transcription while a drastic repression of genes observed were involved in translation, ribosomal structure and biosynthesis followed by those whose products were of unknown function. Since renal involvement during *Brucella* infection is uncommon, the identified genes in the present study could provide additional insights on the strategy of *B. abortus* in their virulence and survival within non-phagocytic cells. Furthermore, genes with unknown function that represent 22% of the differentially highly expressed genes at >7-fold deserve some special attention as this group might contain some potential unknown virulence factors utilized by *Brucella* in their survival within the host.

Conflict of Interest

The authors have no conflict of interest to declare.

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