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Original article

Transcriptomic analysis of *Ehrlichia ruminantium* during the developmental stages in bovine and tick cell culture

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ABSTRACT

The use of bioinformatics tools to search for possible vaccine candidates has been successful in recent years. In an attempt to search for additional vaccine candidates or improve the current heartwater vaccine design, a genome-wide transcriptional profile of *E. ruminantium* (Welgevonden strain) replicating in bovine endothelial cells (BA886) and *Ixodes scapularis* embryonic tick cells (IDE8) was performed. The RNA was collected from the infective extracellular form, the elementary bodies (EBs) and vegetative intracellular form, reticulate bodies (RBs) and was used for transcriptome sequencing. Several genes previously implicated with adhesion, attachment and pathogenicity were exclusively up-regulated in the EBs from bovine and tick cells. Similarly, genes involved in adaptation or survival of *E. ruminantium* in the host cells were up-regulated in the RBs from bovine cells. Thus, it was concluded that those genes expressed in the EBs might be important for infection of mammalian and tick host cells and these may be targets for both cell and humoral mediated immune responses. Alternatively, those exclusively expressed in the RBs may be important for survival in the host cells. Exported or secreted proteins exclusively expressed at this stage are ideal targets for the stimulation of cytotoxic T-lymphocyte (CTL) immune responses in the host.

1. Introduction

Heartwater is a non-contagious tick-borne disease of domestic ruminants caused by an infection with a bacterium *Ehrlichia ruminantium,* which is an α-proteobacterium in the order Rickettsiales that is transmitted by *Amblyomma* ticks (Allsopp, 2015). This disease poses a major threat to livestock productivity particularly in areas where it is endemic such as sub-Saharan Africa, and has also spread to the eastern Caribbean (Walker and Olwage, 1987). The occurrence of the disease prevents more productive breeds from being introduced into endemic areas, while in the USA the presence of the tick vector means that there is a continual threat of the disease being introduced at some stage (Barré et al., 1988). Regardless, there is no safe and effective vaccine currently available. The only commercially available heartwater vaccine employs infection with virulent Ball3 strain infected sheep blood followed by antibiotic treatment and this method offers limited protection against several common virulent genotypes (Allsopp, 2015).

Research has shown that bacterial pathogens interrupt the hosts' cellular pathways for survival and replication and in turn, the infected host cells respond to the invading pathogen through cascading changes in gene expression (Humphrys et al., 2013). Thus, understanding these complex processes to identify novel bacterial virulence factors and host immune response pathways remains vital, particularly in vaccine development (Allsopp, 2015). The molecular mechanisms by which *E. ruminantium* proteins manipulate the host thereby facilitating infection have not been well defined. We therefore hypothesized that those genes highly expressed in the elementary bodies (EBs) from bovine cells (BovEBs) and tick cells (TicEBs) may be important for infection of mammalian and tick host cells. Similarly, those expressed in the reticulate bodies (RBs) may be central for survival in the host cells. Since this study was used as a platform to select for promising vaccine candidates, our focus was mainly on the genes from these functional

Abbreviations: BA886, Bovine aorta endothelial cell lines; BovEBs, Bovine cell derived EBs; BovRBs, Bovine cell derived RBs; cDNA, Complementary DNA; Ct, Threshold value; CTL, Cytotoxic T-lymphocytes; DNA, Deoxyribonucleic acid; EB, Elementary body; EDGE, Empirical analysis of digital gene expression; FC, Fold change; IDE8, *Ixodes scapularis* embryonic tick cell line; IFN, Interferon; NGS, Next generation sequencing; Pi, Post infection; qPCR, Quantitative PCR; RB, Reticulate body; RNA, Ribonucleic acid; rRNA, ribosomal RNA; RNA-seq, RNA sequencing; RPKM, Reads per kilo base of gene model per million mapped reads; RT-qPCR, Reverse transcription qPCR; T4SS, Type IV secretion system; Th1, T-helper 1; tRNA, transfer RNA; TicEBs, Tick cell derived EBs; tmRNA, Transfer messenger RNA; TR, Tandem repeats; TRPs, Tandem repeat proteins

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The sequential development of *E. ruminantium* has been described both in vertebrate and invertebrate hosts (Allsopp, 2015). *In vitro* studies revealed that *E. ruminantium* has a complex life cycle that is described as *Chlamydia*-like developmental stages (Jongejan et al., 1991). The *E. ruminantium* life cycle has the smaller extracellular EBs that are infectious but not replicative and the larger intracellular replicative and non-infectious RBs (Jongejan et al., 1991). In the early stages, the EBs adhere to the host target cells and are immersed quickly. These persist within the intracytoplasmic vacuoles where they divide by binary fission to form vegetative forms, RBs and 2–4 days post infection (pi), the intermediate bodies are formed. After 5–6 days the host cells are disrupted and EBs are released and thus initiating a new cycle of infection (Jongejan et al., 1991).

Global analysis of bacterial gene expression has previously been hindered by several factors, some of which include high abundance of ribosomal RNA and/or RNA instability (Neidhardt and Umbarger, 1996; Filiatrault, 2011). Furthermore, mRNA enrichment has been challenging in previous years (Rossetti et al., 2010). However, with the advent of new technology such as next generation sequencing (NGS) in combination with mRNA enrichment and tilling array technology, it has become practicable to understand and analyse the bacterial transcriptome (Sorek and Cossart, 2010). NGS allows an opportunity to obtain millions of reads at a low cost and has opened the door to study microorganisms that cannot be easily purified (Wang et al., 2009). To our knowledge, this is the first study to investigate the global gene expression analysis of *E. ruminantium* Welgevonden in different developmental stages using transcriptome or RNA-sequencing. Available studies are based on microarray analysis (Emboulé et al., 2009; Pruneau et al., 2012). In general, microarray results are consistent with RNA-seq data, but the RNA-seq technique is more sensitive (Wang et al., 2009). Microarray-based techniques require knowledge of the genome sequence and high background cross hybridisation occurs. Additionally, comparisons of expression levels across different experiments are difficult and requires complex normalisation. Transcriptome sequencing on the other hand, offers an advantage over previously described methods for studying bacterial gene expression in that it provides a more precise measurement of transcripts and their isoforms (Wang et al., 2009). Generally, the extracted RNA from infected cells is a mixture of host and bacterial RNA. Whereas most of the bacterial RNA is ribosomal RNA (rRNA) and tRNA (up to 98%), bacterial mRNA is a typical minor fraction of the infected cells (Humphrys et al., 2013). Thus, RNA-seq offers advantages in that it is sensitive, transcripts can be accurately quantitated and it is not limited to detect transcripts that correspond to the existing genome sequence. This study reports that several *E. ruminantium* genes were differentially expressed in the developmental stages *in vitro* in mammalian and tick cells. The RNA-seq data was validated with reverse transcription quantitative real time (RT-qPCR).

2. Materials and methods

2.1. Strain of E. ruminantium

The *E. ruminantium* Welgevonden strain was originally isolated from a mouse infected with a tick homogenate. This male tick of *Amblyomma hebraeum* was collected on the Welgevonden farm (Naboomspruit/Mokgopong) in the Northern Transvaal (Limpopo province) (Du Plessis, 1985). The EBs of the *E. ruminantium* Welgevonden strain, obtained as passage (179), were stored in 500 µl sucrose-potassium phosphate glutamate medium (SPG) (0.218 M sucrose, 3.8 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM $C_5H_8NO_4K$) and frozen in liquid nitrogen.

2.2. Cell lines

Bovine aorta endothelial cells (BA886) were used as mammalian host cells and *Ixodes scapularis* embryonic tick cells (IDE8) as vector host cells for culture of *E. ruminantium*. BA886 cells were propagated in the media containing Dulbecco's modified eagle's medium/Ham's nutrient mixture: F12 (1:1 DME/F12) (Sigma-Aldrich) with 10% fetal bovine serum (FBS) (Life technologies), 1.2 g/L sodium bicarbonate and 100 IU penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The BA886 cells were cultured as monolayers at 37 °C in a SHEL-LAB CO_2 water-jacked incubator (SHEL-LAB). IDE8 cells were propagated in L-15 B media (Munderloh and Kurtti, 1989) with 5% FBS (Life technologies), 10% tryptose phosphate broth (TPB) (Sigma-Aldrich), 0.1% bovine lipoprotein concentrate (MP Biomedicals), 2 mM L-glutamine (Sigma-Aldrich) and 100 IU penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The tick cells were cultured as monolayers at 32 °C in a Labtech incubator (Daihan LabTech). The tick cells were kindly provided by Dr Lesley Bell-Sakyi from the Tick Cell Biobank, Pirbright Institute, UK.

2.3. In vitro infection of cell monolayers with E. ruminantium (Welgevonden) strain

The BA886 cells were infected as described previously (Zweygarth et al., 1997). Briefly, the BA886 cells were inoculated with the supernatant containing the EBs of *E. ruminantium* Welgevonden (passage 179). The EBs were collected after 5–6 days pi in the supernatant when 90% of the cells were infected and the EBs could be visualized extracellular by Kyro-Quick stain (KYRO). The EBs were purified for transcriptome sequencing as follows: Any intact host cells were lysed by 2 passages through a 25-gauge needle syringe (Terumo Medicals) and host cell debris removed from the EBs by centrifugation at 1 500 xg for 10 min. The supernatant was transferred to a new microcentrifuge tube and the EBs were collected by centrifugation at 20 000 xg for 30 min. The pellet was resuspended in 1 ml TRI Reagent (Sigma-Aldrich) for RNA extraction according to the manufacturer's instructions.

For the isolation of RBs embedded in the BA886 cells, a new batch of confluent bovine endothelial cells (BA886) were infected. To remove all EBs from the inoculum, all the media was discarded after 24 h and replaced with 5 ml fresh medium. In addition, the level of infectivity was monitored every 6 h by microscopy. When the RBs were observed, the medium was discarded and the infected endothelial cells were washed twice with 2 ml Dulbecco's PBS (Sigma-Aldrich) and fresh media added. Once 70–80% host cells contained RBs (2–3 days pi), the cells were harvested and centrifuged at 1500 xg for 10 min. The supernatant was discarded and the pellet (containing RBs) was collected and resuspended in 1 ml TRI Reagent.

The IDE8 cells were inoculated with the supernatant containing the EBs of *E. ruminantium* (Welgevonden) that were collected from previously infected bovine endothelial cells as previously described (Bell-Sakyi et al., 2000). The inoculated tick cells were maintained with weekly medium changes. The cultures were monitored for growth and infection by weekly preparation of cytospin Kyro-quick (KYRO) stained smears (Zweygarth et al., 1997). Cultures that were not infected after 12–14 weeks were discarded. The EBs were collected as described for the BA cells.

2.4. Transcriptome sequencing

RNA was processed from two biological replicates of *E. ruminantium* EBs from ticks (TicEBs), bovine EBs (BovEBs) and bovine RBs (BovRBs). The TRI Reagent (Sigma-Aldrich) protocol was used for RNA extraction. The Ribo-Zero™ magnetic kit (Gram negative bacteria, Epicentre, Illumina) was used to remove ribosomal RNA. The rRNA depleted samples were further purified using the RNeasy MiniElute Cleanup kit (Qiagen) according to the manufacturer's instruction. The RNA libraries