Veterinary Parasitology 212 (2015) 111–117

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Comparative RNA-seq analysis of the *Tritrichomonas foetus* PIG30/1 isolate from pigs reveals close association with *Tritrichomonas foetus* BP-4 isolate 'bovine genotype'

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ARTICLE INFO

Article history:
Received 19 June 2015
Received in revised form 11 August 2015
Accepted 12 August 2015

Keywords:
Trichomonosis
Cow
Pig
Cat
Cysteine protease
RNA sequencing
Transcriptome

ABSTRACT

Tritrichomonas foetus was described as a commensal of the stomach, caecum and nasal cavity of pigs before it was recognised as the cause of reproductive tract disease of cattle. *T. foetus* also causes chronic large bowel diarrhoea in domestic cats. Multi-locus genotyping and comparative transcriptome analysis has previously revealed that *T. foetus* isolated from cat and cattle hosts are genetically distinct, referred to as the 'feline genotype' and 'bovine genotype', respectively. Conversely, multi-locus genotyping has grouped porcine *T. foetus* with the 'bovine genotype'. To compare the extent of the similarity between porcine *T. foetus* and cattle 'bovine genotype' isolates, RNA-sequencing (RNA-seq) was used to produce the first cell-wide transcriptome library of porcine *T. foetus* PIG30/1. Comparative transcriptome analysis of the PIG30/1 with the published bovine (BP-4) and feline (G10/1) transcriptomes revealed that the porcine *T. foetus* shares a 4.7 fold greater number of orthologous genes with the bovine *T. foetus* than with the feline *T. foetus*. Comparing transcription of the virulence factors, cysteine proteases (CP) between the three isolates, the porcine *T. foetus* was found to preferentially transcribe CP8 like the 'bovine genotype' *T. foetus,* compared to thehigh transcription of CP7 seen for 'feline genotype' *T. foetus.* At the cell-wide transcriptome level, the porcine *T. foetus* isolate (PIG30/1) groups closer with the 'bovine genotype' *T. foetus* rather than the 'feline genotype' *T. foetus.*

1. Introduction

Isolates of the flagellate *Tritrichomonas foetus* that reside in the stomach, caecum and nasal cavity of pigs are not of clinical significance to their porcine hosts (Fitzgerald et al., 1958; Hammond et al., 1958; Hibler et al., 1960; Pakandl, 1994; Mostegl et al., 2011). The same species is a serious urogenital tract parasite of cattle with transmission occurring via mating of infected, asymptomatic bulls to females. Although now considered a rare disease as a result of artificial insemination, bovine trichomonosis causes abortion and infertility in infected female cattle (Riedmuller, 1928; Parsonson et al., 1976; Rhyan et al., 1988; Rae et al., 2004). More recently, *T. foetus* is globally recognized as the cause of chronic large bowel diarrhoea in domestic cats (Levy et al., 2003; Bell et al., 2010; Lim et al., 2010). While *T. foetus* is not known to have environmentally resistant cysts that facilitate transmission, cross-species infection has been suggested (Fitzgerald et al., 1958). Indeed, experimental infections of *T. foetus* in their non-respective hosts have shown that the porcine *T. foetus* isolate is able to cause disease in cattle (Fitzgerald et al., 1958), and similarly the feline and bovine isolates have been shown to cause disease in their non-respective hosts (Stockdale et al., 2007; Stockdale et al., 2008). Recently, however, a high prevalence of *T. foetus* in pigs farmed in close proximity with *T. foetus*-free cattle has been shown, implying that the risk of cross-infections of *T. foetus* from pigs to cattle is negligible (Mueller et al., 2015).

Owing to the broad host range and organ tropism of *T. foetus,* much emphasis has been placed on identifying the difference, if any, between host-specific isolates. Various molecular methods including multi-locus genotyping have grouped the porcine *T. foetus* isolate with bovine *T. foetus* isolates, whereas isolates of *T. foetus* from cats are genetically distinct from bovine isolates (Tachezy et al., 2002; Šlapeta et al., 2010; Šlapeta et al., 2012). In an accompanying study by Mueller et al. (2015), the porcine isolate PIG30/1 was shown to be identical to the 'bovine genotype' of *T. foetus* at 9 diagnostic molecular markers. Whole genome dif-

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http://dx.doi.org/10.1016/j.vetpar.2015.08.012
0304-4017/

Table 1
Summary statistics of the *Tritrichomonas foetus* (PIG30/1) transcriptome.

	Summary statistics for *T. foetus* RNA sequencing		
Feature	Porcine PIG30/1[a]	Bovine BP-4[b]	Feline G10/1[b]
Total number of reads	55,506,706	64,744,882	64,009,804
Total base pairs (nt)	5,606,177,306	6,539,233,082	6,464,990,204
Total number of assembled contigs	43,308	42,363	36,559
Total assembled bases (nt)	47,094,268	37,882,427	29,525,551
Mean length of contigs(nt)	1,087	895.25	806.61
%GC content in transcriptome	33.90	34.62	34.87
Minimum contig length (nt)	201	201	201
Maximum contig length (nt)	17,203	14,314	17,195
Contig N50	1,503	1,259	1,178

[a] Summary statistics of the porcine *T. foetus* transcriptome sequenced in this study.
[b] Summary statistics of the bovine and feline *T. foetus* transcriptome sequenced by Morin-Adeline et al. (2014) for comparison. Paired-end RNA sequencing of the bovine and feline *T. foetus* transcriptomes were performed on an Illumina HiSeq2000 platform and assembled using the same *de novo* approach as for the porcine *T. foetus* transcriptome in this study.

ferences between the 'feline genotype' and the 'bovine genotype' are minute and analysis of 1511 orthologous protein-coding genes shared between a bovine (BP-4) and a feline (G10/1) isolate indicate little divergence, despite their vastly different origin (. at 9 diagnostic molecular markers. Morin-Adeline et al., 2014). In addition, differences in specific virulence factors have been investigated at a cell-wide level in an attempt to understand molecular mechanisms that govern host-specificity of the different isolates (Šlapeta et al., 2010, 2012; Morin-Adeline et al., 2014). In particular, the cysteine protease (CP) gene family have been used as they are regarded as a crucial aspect of *T. foetus* virulence involved in cleavage and inactivation of host protective antibodies (Bastida-Corcuera et al., 2000). Distinct differences exist in the cysteine protease gene family between the bovine and feline genotype *T. foetus* and to date, 7CPs have been used to as molecular diagnostic markers to distinguish the 'bovine genotype' from the 'feline genotype' (Šlapeta et al., 2012). The most divergent of the family, CP2, confirmed that *T. foetus* isolate (PIG30/1) is 'bovine genotype' (Mueller et al., 2015).

The apparent identity of the porcine and bovine isolates of *T. foetus* compared to the feline isolate of *T. foetus*, together with their broad host range, presents an intriguing model to studying the factors that drive *T. foetus* host adaptation. Comparative analysis of transcriptomes offers an ideal method for whole-cell comparisons in the absence of a sequenced nuclear genome (Morin-Adeline et al., 2014a). In this study, RNA-sequencing (RNA-seq) was utilised to obtain whole-cell transcribed sequences of the novel PIG30/1 porcine *T. foetus* isolated by Mueller et al. (2015). The newly assembled transcriptome of PIG30/1 was then compared to the published bovine (BP-4) and feline (G10/1) *T. foetus* (Morin-Adeline et al., 2014).

2. Material and methods

2.1. Parasite culture

The porcine *T. foetus* PIG30/1 isolate used for this study was isolated from pig faeces at The University of Sydney May Farm, Camden, New South Wales, Australia (Mueller et al., 2015). Axenic cultures of the parasite were maintained in Modified Diamond's Medium (MDM) (200 ml) prepared with 4 g Trypticase peptone (BD211921; Becton, Dickinson and company, NSW, Australia), 2 g yeast extract (BD211929; Becton, Dickinson and company, NSW, Australia) and 1 g maltose (BD216830; Becton, Dickinson and company, NSW, Australia) at pH 7.2 (ATCC Medium 719 without agar; http://www.atcc.org/) and supplemented with 10% inactivated sheep serum (Life Sciences, Australia). The antibiotics PenStrep & Fungizone was added to the media to protect against bacterial contamination at a final concentration of 10 U/ml penicillin, 10 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco 15,240,062, Life Sciences, Australia). All cultures were maintained in sterile 15 ml glass bijou bottles and sub-cultured daily.

2.2. Total RNA isolation

Trophozoites of PIG30/1 *T. foetus* at mid-exponential phase in culture were collected and 10^7 cells were pelleted at $3220 \times g$ for 5 min and prepared for RNA-seq (Morin-Adeline et al., 2014). RNA isolation was carried out using the RNeasy Micro kit (Qiagen, New South Wales, Australia) according to the manufacturer's instructions. Homogenisation was carried out in a FastPrep®-24 high-speed homogeniser (MP Biomedicals, USA) for 30 s at 4 m/s. An in-column DNAase (Sigma–Aldrich) treatment step was included, which was incubated at room temperature for 15 min. RNA was eluted in 30 μl of sterile water, assessed for quality and quantity using a 2100 Bioanalyzer (Agilent Technologies, Inc) and preserved in an RNAstable® tube (Biometrica) by drying in a Savant SpeedVac (ThermoFisher, Australia) concentrator connected to a vapor trap for 1 h. Paired-end RNA sequencing on the Illumina HiSeq2000 platform was performed by Macrogen (Seoul, Korea).

2.3. Transcriptome assembly, annotation and identification of shared transcripts between T. foetus isolates

The quality of raw sequence reads was confirmed using FASTQC (Babraham Bioinfomatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) prior to assembly. A *de novo* transcriptome assembly approach was adopted using the default parameters of Trinity within the Galaxy suite platform to assemble right and left reads, resulting in the porcine *T. foetus* transcriptome (PIG30/1) (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). Sequences were initially annotated using BLASTX against the NCBI non-redundant (nr) database with an e-value cut-off of 1×10^{-3} implemented in the Galaxy Suite (Camacho et al., 2009; Cock et al., 2013). Functional annotation of sequences at the gene ontology (GO) level was carried out using the Blast2GO platform (version 3.0) abiding to default parameters (Conesa et al., 2005). Combined graphs were generated for cellular component, biological processes and molecular function with a level 3 cut-off and filtered to a minimum of 100 sequences per GO category. The GO-enzyme code mapping function in Blast2GO, which maps GO terms to enzyme codes, as well as a search of enzyme pathways from the online Kyoto Encyclopedia of Genes and Genomes (KEGG), was implemented using default settings.

To identify putative shared orthologue transcripts between the bovine, feline and porcine *T. foetus* isolates, a reciprocal BLAST method was adopted in the Galaxy Suite platform (Giardine et al.,