RNA sequencing differential gene expression analysis of isolated perfused bovine udders experimentally inoculated with *Streptococcus agalactiae*

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ABSTRACT

The aim of this study was to elucidate the differential gene expression in the RNA sequencing transcriptome of isolated perfused udders collected from 4 slaughtered Holstein × Zebu crossbred dairy cows experimentally inoculated with *Streptococcus agalactiae*. We studied 3 different statistical tools (edgeR, baySeq, and Cuffdiff 2). In summary, 2 quarters of each udder were experimentally inoculated with *Strep. agalactiae* and the other 2 were used as a control. Mammary tissue biopsies were collected at times 0 and 3 h after infection. The total RNA was extracted and sequenced on an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA). Transcripts were assembled from the reads aligned to the bovine UMD 3.1 reference genome, and the statistical analyses were performed using the previously mentioned tools (edgeR, baySeq, and Cuffdiff 2). Finally, the identified genes were submitted to pathway enrichment analysis. A total of 1,756, 1,161, and 3,389 genes with differential gene expression were identified when using edgeR, baySeq, and Cuffdiff 2, respectively. A total of 122 genes were identified by the overlapping of the 3 methods; however, only the platelet activation presented a significantly enriched pathway. From the results, we suggest the *FCER1G*, *GNAI2*, *ORAI1*, and *VASP* genes shared among the 3 methods in this pathway for posterior biological validation.

Key words: baySeq, crossbred dairy cow, Cuffdiff 2, edgeR

INTRODUCTION

The identification of genes expressed in different tissues leads to a better understanding of molecular mechanisms that are relevant for animal production.

Ribonucleic acid sequencing (RNA-seq) allows a wider gene expression analysis because the total RNA from a given tissue can be sequenced (Wang et al., 2009). Different statistical methods are available for performing RNA-seq differential gene expression analysis in a range of biological experiments (Hardcastle and Kelly, 2010; Robinson et al., 2010a).

The edgeR (Robinson et al., 2010a) and baySeq (Hardcastle and Kelly, 2010) packages from Bioconductor/R software and the Cuffdiff 2 (Trapnell et al., 2013) package implemented in Cufflinks software have been widely used to perform statistical analysis of RNA-seq data (Soneson and Delorenzi, 2013; Seyednasrollah et al., 2015). These tools were proposed to detect differential gene expression based on different statistical assumptions, leading to distinct results. Thus, the evaluation of all these methods in RNA-seq experimental designs is recommended (Seyednasrollah et al., 2015). This evaluation is used to improve the selection of candidate genes for posterior biological validation based on pathway enrichment analysis. The aim of this study was to elucidate the differential gene expression in the RNA-seq transcriptome of isolated perfused udders, collected from Holstein × Zebu crossbred dairy cows experimentally inoculated with *Streptococcus agalactiae*, using the previously mentioned statistical methods.

MATERIALS AND METHODS

All animal care and handling procedures followed regulations approved by the animal research ethics committee of Embrapa Dairy Cattle (Juiz de Fora, MG, Brazil; license no. 11/2011).

Data Collection

The samples were obtained from 4 Holstein × Zebu crossbred dairy cows slaughtered in the experimental center “Campo Experimental José Henrique Bruschi” at Embrapa Dairy Cattle. Their udders were extracted

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and kept in an isolated perfused system. Two quarters of each udder were experimentally inoculated with *Strep. agalactiae*, and the other 2 quarters were used as a control. Mammary tissue biopsies were collected at times 0 and 3 h after infection.

RNA Extraction

The RNA extraction was performed in the Molecular Genetics Laboratory at Embrapa Dairy Cattle. The total RNA was extracted in 2 replicates using the RNeasy Mini Kit (Qiagen, Valencia, CA). The protocol is based on cell lysis, followed by a DNase treatment to avoid DNA contamination, binding of RNA to the silica gel membrane, purification, and subsequent elution of total RNA. The total isolated RNA was quantified by spectrophotometry (NanoDrop, Wilmington, DE), and its integrity was evaluated by RNA integrity number score on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The average RNA integrity number $\pm$ standard deviation was 6.69 ± 0.46 . For each udder, a pool of RNA sample from the 2 inoculated quarters was obtained and another pool of RNA sample was collected from the 2 control quarters. These collections were made 2 times (0 and 3 h) for all 4 animals, totaling 16 samples (4 biological replicates $\times$ 2 collections $\times$ 2 conditions).

RNA Sequencing

The RNA was sequenced by a commercial provider that prepared cDNA libraries using a TruSeq RNA sample preparation kit (Illumina, San Diego, CA), generating about 19 million 100-bp reads. The libraries were quantified by real-time PCR and sequenced (2 $\times$ 100 paired-end reads) on a HiSeq 2000 sequencer (Illumina) using the TruSeq SBS Kit version 3 (Illumina).

Quality Control and Alignment

Quality control was performed using FastQC (version 0.65; Afgan et al., 2016; <https://usegalaxy.org/>). Adapter sequences were removed from reads; however, low-quality or ambiguous reads were not trimmed be-

cause a preliminary test showed that they did not align to the reference genome.

Reads were mapped to the bovine genome UMD 3.1 assembly (<http://www.ncbi.nlm.nih.gov/genome?term=bos%20taurus>) using TopHat (version 2.1.0; Trapnell et al., 2009) with default settings and following the authors' protocol. The unmapped reads were subjected to a BLAST search on a nonredundant protein database using NCBI BLAST + blastx (version 0.1.07; Camacho et al., 2009).

Following the alignment, Cufflinks (version 2.2.1.0) with default settings (Trapnell et al., 2010) was used to assemble the transcripts. The abundance of the transcripts was reported by Cufflinks as fragments per kilobase of transcript per million fragments sequenced, which was the normalized expression level, later used by the Cuffdiff 2.

Gene expression was then calculated by counting the reads mapped to exons using htseq-count (version 0.6.1; Anders et al., 2015). The normalized gene expression data were summarized in an expression matrix, which was later used by edgeR and baySeq. A multi-dimensional scaling method was used to represent the similarities between samples because it reproduces the observed distance matrix in a space with a reduced number of chosen dimensions (Hair et al., 2009).

Statistical Analyses

Gene expression was compared between control and inoculated samples at times 0 and 3 h after inoculation using 3 statistical methods (Table 1). The version of the gene model used for this study was Ensembl Gene 92 (Aken et al., 2016). Three different tools were used, and these were chosen to study distinct and nonoverlapping methodologies.

EdgeR Tool. The method implemented in the edgeR package (Galaxy tool version 0.0.2; Robinson et al., 2010a) uses the quantile-adjusted conditional maximum likelihood procedure (Robinson and Smyth, 2008) in an exact test similar to the Fisher exact test to determine differential gene expression for pairwise comparisons. It assumes a negative binomial (NB) distribution for the data in a balanced incomplete block design as follows:

Table 1. Description of the statistical assumptions used in the methods for RNA sequencing differential gene expression analysis

Method ¹	Normalization ²	Distribution	Differential expression test
edgeR	TMM	Negative binomial	Exact test
baySeq	RLE	Negative binomial	Posterior probabilities
Cuffdiff 2	Geometric classified by FPKM	β -negative binomial	<i>t</i> -test

¹edgeR: Robinson et al. (2010a). baySeq: Hardcastle and Kelly (2010). Cuffdiff 2: Trapnell et al. (2013).
²TMM = trimmed mean of *M*-values; RLE = relative log expression; FPKM = fragments per kilobase of transcript per million fragments sequenced.

$$Y_{gi} \sim \text{NB}(M_i p_{gj}, \phi_g),$$

where g is the gene and i is the sample, M_i is the library size (total number of reads), ϕ_g is the dispersion, and p_{gj} is the relative abundance of the gene g in the experimental group j to which the sample i belongs. The NB parameterization where the mean was $\mu_{gi} = M_i p_{gj}$ and the variance was $\mu_{gi}(1 + \mu_{gi} \phi_g)$ was used. The normalization factor named trimmed mean of M -values was estimated between each pair of samples (Robinson and Oshlack, 2010b). Individual gene expression was calculated as the mean expression of each gene averaged over all samples of each group and presented as the logarithm of counts per million reads. The Benjamini-Hochberg procedure, proposed by Benjamini and Hochberg (1995), was used to control the false discovery rate (**FDR**), and a cut-off criterion of FDR-adjusted $P < 0.01$ was applied to identify differentially expressed genes.

BaySeq Tool. The method applied in the baySeq R/Bioconductor package (version 1.16.0; Hardcastle and Kelly, 2010) uses prior information to fit a nonlinear model that quantifies biases and uncertainties in the estimates (Liu and Logvinenko, 2003). It also assumes an NB distribution for the data and derives an empirically determined prior distribution from the entire data set by maximum likelihood (Hardcastle and Kelly, 2010), represented as follows:

$$Y_{ij} \sim \text{NB}(M_{qij}, \phi_q),$$

where Y_{ij} is the number of reads for the gene i in the j th sample, M_{qij} is the mean, and ϕ_q is the dispersion. The counting data from the data set with $n = 16$ samples $A = \{A_1, \dots, A_{16}\}$ are given by $(u_{ic}, \dots, u_{nc})$, where u_{ic} is the counting for a given gene c in sample i . For each sample A_i , the library size was obtained from a scale factor l_i . Thus, for each gene (c), the data (D) were considered as follows:

$$D_c = [(u_{ic}, \dots, u_{nc}), (l_1, \dots, l_{16})].$$

Two models were defined. In the first model, the entire data set, inoculated (I) and control (C), was considered as a single treatment:

$$D_{PC} = [(u_{I1}, u_{I2}, u_{I3}, u_{I4}, u_{C1}, u_{C2}, u_{C3}, u_{C4}), (l_{I1}, l_{I2}, l_{I3}, l_{I4}, l_{C1}, l_{C2}, l_{C3}, l_{C4})],$$

whereas in the alternative model, the differences between inoculated (I) and control (C) data sets were considered:

$$D_P = [(u_{I1}, u_{I2}, u_{I3}, u_{I4}), (l_{I1}, l_{I2}, l_{I3}, l_{I4})]$$

$$D_C = [(u_{C1}, u_{C2}, u_{C3}, u_{C4}), (l_{C1}, l_{C2}, l_{C3}, l_{C4})].$$

Last, a normalization factor named relative log expression, described by Anders and Huber (2010), was used to compare both models. Posterior probabilities and FDR for each gene were calculated instead of significance values. A cut-off criterion of posterior probability < 0.01 was applied to identify differentially expressed genes.

Cuffdiff 2 Tool. The Cuffdiff 2 method (Trapnell et al., 2013), implemented in Cufflinks software (Trapnell et al., 2010), was used with default settings. It uses a model for read counts based on a β -NB distribution and a t -test to identify differentially expressed genes and allows differential gene expression analysis between treatment and control based on a pairwise analysis without replicates. A geometric normalization procedure, classified by fragments per kilobase of transcript per million fragments sequenced, was applied to determine the minimum number of reads needed to reconstruct the entire sequence. Assumptions are

$$x_t^j \sim \text{BNB}(\mu_t^j, \sigma_t^{j^2}),$$

where x_t^j is the number of fragments in the replicate j from the transcript t , BNB is the β -NB, μ is the mean, and σ^2 is the variance. The Benjamini-Hochberg procedure was used as previously described in the baySeq method section.

Identification of Metabolic Pathways

The differentially expressed genes identified by each of the 3 methods were analyzed independently using DAVID Bioinformatic Resources 6.8 (Huang et al., 2009a,b), and pathways with $P < 0.05$ were considered enriched.

RESULTS AND DISCUSSION

The total and the average number of reads generated by the RNA-seq were 842,604,108 and 26,331,378, respectively. The quality control performed using FastQC provided an overview of the variation in base quality scores at each position of the input file. The samples were of good quality based on the Phred scores (probability of incorrect base call).

Given the good quality of the cDNA libraries and the fact that mapping algorithms use only reads that perfectly match a position on the reference genome, the analyses were carried out without trimming the reads. On average, 88.91 (Table 2) and 88.89% of the

Table 2. Total and proportion of aligned reads per sample

Treatment and sample label ¹	Left reads		Right reads		Aligned pairs	
	Total no.	%	Total no.	%	Total no.	%
Control						
G7T0	30,692,458	89.9	30,419,535	89.1	29,164,959	84.2
G7T3	23,756,098	87.9	23,934,459	88.6	22,638,978	82.6
G8T0	21,164,058	90.4	21,137,250	90.3	20,248,224	85.3
G8T3	23,096,338	89.0	23,253,768	89.6	21,943,088	83.9
G9T0	23,054,518	87.5	23,235,820	88.2	21,811,906	82.3
G9T3	22,784,883	90.2	22,690,679	89.9	21,607,226	85.0
G10T0	21,290,821	88.6	21,216,345	88.3	20,123,296	82.6
G10T3	19,639,786	89.2	19,736,210	89.7	18,642,344	83.9
Inoculated						
G7T0	22,510,799	86.3	22,658,924	86.9	21,280,138	80.9
G7T3	23,227,834	89.8	23,142,353	89.5	22,129,511	84.2
G8T0	21,707,055	88.3	21,809,049	88.7	20,653,152	82.8
G8T3	22,543,993	89.5	22,395,590	88.9	21,408,569	83.8
G9T0	33,428,420	90.4	33,434,970	90.4	31,943,198	85.1
G9T3	22,334,160	88.4	22,479,819	89.0	21,294,357	83.2
G10T0	21,647,272	86.5	21,777,734	87.0	20,470,883	80.5
G10T3	21,589,981	89.5	21,473,579	89.0	20,466,780	83.8

¹G7 to G10: sample identification; T0 and T3: time (h) after inoculation.

trimmed and nontrimmed reads, respectively, were mapped to the reference genome. This difference is due to the lower number of reads in the trimmed data set. Similar results were reported by Cui et al. (2014), who also aligned sequenced mammary tissue samples using TopHat.

The unmapped reads subjected to the BLAST search were aligned to other members of the Bovidae family, and several of these regions are related to immune system processes, suggesting that the alleles are highly polymorphic and have not yet been annotated in the *Bos taurus* genome. During the multidimensional scaling analysis, the control sample G7T3 was clustered together with the inoculated samples (Figure 1), and it was removed from further analysis.

There are 26,453 annotated genes in the UMD 3.1 bovine genome assembly. A total of 27,404 sequences were expressed in the mammary tissue, of which 21,691 were annotated genes. The remaining 5,821 sequences were labeled as XLOC. A larger number of differentially expressed genes was reported by Cuffdiff 2, followed by edgeR and baySeq (Table 3).

The variation in the number of differentially expressed genes is explained by the different statistical assumptions that each method is based on (Table 1). The different normalization methods that are used for these analyses may be largely responsible for the variability in the results, having more of an effect on the detection of genes differentially expressed than the statistical test used (Bullard et al., 2010; Maza et al., 2013).

The fragments per kilobase of transcript per million fragments sequenced (FPKM) normalization method is associated with the greatest number of false dis-

coveries compared with relative log expression and trimmed mean of *M*-values methods; this also occurs in the production of mean squared errors, so the FPKM normalization method may not be the most suitable for differential gene expression analyses (Maza et al., 2013). The trimmed mean of *M*-values normalization method is satisfactory for maintaining a reasonable false-positive rate without any loss of power (Dillies et al., 2013) and performs very similar to relative log

Figure 1. Multidimensional scaling plot of the samples in which distances correspond to normalized gene expression between pairs of samples. G7 to G10 = sample identification; T3 = time 3 h after inoculation; ■ = inoculated; ● = control.

expression, once both can provide equal results in some simpler experiments (Maza et al., 2013; Maza, 2016). These methods perform well for the analysis of differentially expressed genes (Maza et al., 2013).

The baySeq assumes an NB distribution; however, it estimates posterior probabilities and the parameter distributions based on an empirical Bayesian method (Hardcastle and Kelly, 2010). Sonesson and Delorenzi (2013) and Rapaport et al. (2013) observed that baySeq was computationally slower but had a better control of FDR with a large sample size and no outliers (Sonesson and Delorenzi, 2013). In addition, baySeq it is known to be more conservative than the other methods (Rapaport et al., 2013; Sonesson and Delorenzi, 2013; Seyednasrollah et al., 2015), which was corroborated in this study by the lower number of differentially expressed genes identified (1,161).

In contrast, Cuffdiff 2 is considered less conservative and reported to have a higher FDR (Rapaport et al., 2013). The fact that the method is less conservative was also observed in the present study: 3,389 differentially expressed genes were identified by Cuffdiff 2. According to Rapaport et al. (2013), Cuffdiff 2 uses a Poisson model to control variability in sequencing depth, biological noise, and splicing structure by calculating the mean count across replicates and *P*-values for any observed changes in a transcript's fragment count (Trapnell et al., 2013). The traditional statistical methods, as *t*-test, cannot be reliably applied to genomic data (Law et al., 2014). This method makes assumptions of normally distributed continuous data, whereas the reads are count based and cannot be normally distributed.

In the present study, the number of differentially expressed genes identified using edgeR was intermediary (1,756) between the other 2 methods. The edgeR implements a frequentist method that uses an empirical Bayes procedure to adjust the overdispersion across genes by using the information of other genes (Robinson and Smyth, 2007, 2008). Sonesson and Delorenzi (2013) considered the edgeR to be computationally efficient but less conservative compared with other methods, reporting a larger FDR. Rapaport et al. (2013) also reported variable FDR when comparing edgeR, baySeq,

and Cuffdiff 2; however, both baySeq and edgeR had lower FDR.

The number of differentially expressed genes identified in the present study is in accordance with the literature because other studies have reported more genes detected by Cuffdiff 2, followed by edgeR and baySeq. The present results suggest that baySeq is more conservative than edgeR and Cuffdiff 2 and that the identification of differentially expressed genes by Cuffdiff 2 may be inflated (Table 3). An exception found in the literature, a study by Seyednasrollah et al. (2015), presented Cuffdiff 2 as conservative, reporting lower FDR compared with other methods.

Although the results from the 3 statistical tools were different (Figure 2), they seemed to be suitable for RNA-seq differential gene expression analysis in this study. There were only 122 shared genes among the methods. The most similar results were obtained between edgeR and baySeq, which shared 900 differentially expressed genes. In contrast, there were only 221 shared genes between Cuffdiff 2 and edgeR and only 145 shared genes between Cuffdiff 2 and baySeq.

A total of 5,519 transcripts were reported as differentially expressed, out of which 4,214 were annotated genes in UMD 3.1 genome assembly and were subjected to a pathway enrichment analysis (Figure 3). A total of 8, 8, and 64 pathways were significantly enriched ($P < 0.05$) for edgeR, baySeq, and Cuffdiff 2, respectively. Table 4 presents the shared enriched pathways when studying the results from the 3 statistical methods. The platelet activation (bta04611) was the only pathway found to be enriched by all 3 methods, denoting that these methods resulted in different sets of differentially expressed genes. Four genes [Fc fragment of IgE receptor Ig (*FCER1G*), G protein subunit α i2 (*GNAI2*), ORAI calcium release-activated calcium modulator 1 (*ORAI1*), and vasodilator-stimulated phosphoprotein (*VASP*)] shared among the 3 methods studied were enriched in the pathway platelet activation; therefore, these genes should be indicated for future validation.

Using only the 122 shared differentially expressed genes among the 3 methods (Figure 2) could reduce the false-positive genes in the further analysis, supported by evidence of true differential expression in the tissue.

Table 3. Total and shared differentially expressed genes for the statistical contrasts according to the method used

Contrast ¹	Method ²			Shared genes (no.)
	edgeR	baySeq	Cuffdiff 2	
Inoculated T0 $\times$ control T0	0	0	0	0
Inoculated T3 $\times$ control T3	1,756	1,161	3,389	122

¹T0 and T3: at time 0 and 3 h after inoculation, respectively.

²edgeR: Robinson et al. (2010a). baySeq: Hardcastle and Kelly (2010). Cuffdiff 2: Trapnell et al. (2013).

Table 4. Shared enriched pathways ($P < 0.05$) associated with the differentially expressed gene identified by 3 statistical methods¹

edgeR and baySeq	edgeR and Cuffdiff 2	baySeq and Cuffdiff 2
bta05012: Parkinson's disease bta04611: platelet activation	bta04611: platelet activation bta04310: Wnt signaling pathway bta05323: rheumatoid arthritis bta04610: complement and coagulation cascades	bta04550: signaling pathways regulating pluripotency of stem cells bta04360: axon guidance bta04611: platelet activation

¹edgeR: Robinson et al. (2010a). baySeq: Hardcastle and Kelly (2010). Cuffdiff 2: Trapnell et al. (2013).

However, the genes identified by one method or another are not always false positives because the experimental design can affect the effectiveness of the methods. Such approach would reduce the number of genes and the statistical power of the following enrichment analysis. Thus, using only the shared differentially expressed genes is not of interest for functional enrichment analysis. Instead, the shared genes could be used for validation by real-time PCR.

CONCLUSIONS

The results from the evaluated tools were different given their specific statistical assumptions. The use of the 3 methods for the detection of differentially ex-

pressed genes helped select the genes to be indicated for validation. From the genes identified by the overlapping of the 3 methods, only platelet activation presented a significantly enriched pathway. We indicated the *FCER1G*, *GNAI2*, *ORAI1*, and *VASP* genes shared among the 3 methods in this pathway for posterior biological validation.

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Figure 2. Venn diagram of the shared differentially expressed genes based on RNA sequencing data through 3 statistical methods: edgeR (Robinson et al., 2010a), baySeq (Hardcastle and Kelly, 2010), and Cuffdiff 2 (Trapnell et al., 2013).

Figure 3. Venn diagram of the total number of significantly enriched pathways ($P < 0.05$) through 3 statistical methods: edgeR (Robinson et al., 2010a), baySeq (Hardcastle and Kelly, 2010), and Cuffdiff 2 (Trapnell et al., 2013).

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