Study on the Mechanism of Injury of Chicken Tracheal Epithelium Caused by *Mycoplasma Gallisepticum* Infection

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Abstract

Mycoplasma gallisepticum (MG) is a pathogenic microorganism that is seriously harmful to the poultry industry. It mainly adsorbs on the cilia and mucosa of respiratory epithelial cells, resulting in tracheal mucosal epithelial injury or ciliary shedding, causing chronic respiratory disease (CRD). In order to study the damage of tracheal mucosal epithelium induced by MG infection in chickens and explore its possible mechanism, SPF chicks were challenged with the wild-type *Mycoplasma gallisepticum* strain MG-HY strain. Then, transcriptome sequencing analysis was performed to study the mechanism of MG tracheal mucosal epithelial injury. During infection, MG localizes and proliferates in the chicken trachea, and induces mucosal epithelial damage. A total of 3112 significantly ($P < 0.01$) differentially expressed genes (DEGs) were selected by RNA-seq, including 1646 up-regulated genes and 1466 down-regulated genes. Functional analysis showed increased expression levels of genes involved in immune defense response and mechanical barrier of tracheal mucosa in infected chicks. The expression level of pro-inflammatory cytokines (TNF- α) increased, activating the upstream protein Ras of the ERK-MLCK signaling pathway, Ras causing ERK1/2 phosphorylation levels to rise and MLCK activation, thus causing phosphatization of MLC, and further regulating the expression and mucous distribution of tight junction protein (TJ), leading to tracheal mucosal epithelial injury in chicks. The results of qRT-PCR assay and immunohistochemical analysis were consistent with the results of transcriptome analysis. Overall, our results provide a basis for further investigation of the mechanisms underlying the epithelial damage of the tracheal membrane in chicks caused by MG-infection, contributing to the understanding of how MG affects avian respiratory diseases.

Introduction

Mycoplasma gallisepticum (MG) is one of the main pathogens causing chronic respiratory disease (CRD) in chickens^[1, 2]. Although the lethality is not very high, it has a serious impact on the egg production rate, hatching rate and feed conversion rate of poultry because of its long course of disease and strong pathogenicity. It is currently considered to be one of the *mycoplasmal* diseases leading to the greatest economic losses in poultry farms^[3]. In the feeding process, the symptoms are generally not obvious when this disease is infected alone, or there are only mild respiratory symptoms, but when the feeding and management are improper or mixed with other diseases, such as infectious bronchitis virus, infectious laryngotracheitis virus, avian influenza virus, etc., it can reduce the performance of flocks^[4], cause serious pathological damage, and cause a dramatic increase in the mortality of flocks.

The main site of MG attachment and colonization is the respiratory epithelium, and after colonization, can activate local immune in the trachea mucosa, causing the overactivation of pro-inflammatory cytokines and collecting a strong inflammatory response, resulting in respiratory distress in chickens, and ultimately triggering severe lesions^[5]. This is the key pathological process that causes chronic respiratory disease in chickens. In addition, MG can change the phenotype of adhesion proteins (antigenic variation), avoid the clearance of innate immune defense mechanisms in the host respiratory tract through the

pathway, and play a key role in its pathogenicity and persistence in the host. In vitro chicken embryo tracheal ring studies have revealed that the expression and release of several chemokines and inflammatory cytokines, including IL-18, IL-1 β , TNF- α , IFN- γ and CCL20^[6, 7], which are involved in the inflammatory response to bacterial infection and are highly correlated with mucosal inflammatory lesions. However, the exact mechanism of respiratory mucosal epithelial injury in chickens caused by MG infection still remain unknown.

In recent years, transcriptome sequencing technology has been widely used in the process of gene discovery and gene expression profiling, while transcriptomic analysis of organisms responsive microorganisms provides an effective means to evaluate the pathogenic mechanism of infected hosts^[8]. An increasing number of such transcriptomic studies have been performed in a number of different organisms, allowing a relatively well-understood molecular mechanism and pathway of inflammatory processes caused by bacteria in humans and other mammals. In contrast, in birds, there is less information on the regulation of host gene responses following MG-infection. The aim of this study was to enrich the available data in chickens and to identify genes and pathways involved in mucosal epithelial injury mechanisms (such as: molecular mechanisms regulated by tight junction protein). First, high-throughput sequencing based transcriptomics was applied to explore the comprehensive gene expression profile of the trachea of MG-infection group and control group chickens, with different expression levels DEGs were then functionally annotated and classified, and it was shown that MG-infection can induce the secretion of cytokines (TNF- α) to activate the ERK-MCLK signaling pathway, resulting in tracheal mucosal epithelium damage in chickens. Finally, to further confirm the results of transcriptome analysis, the effect of MG infection on the expression of genes involved in the regulation mechanism of intercellular versus tight junction proteins was analyzed using qRT-PCR and immunohistochemical methods. This study should provide a basis for the further study of the interplay between MG the native host, as well as provide new insights into the mechanism of mucosal epithelial injury triggered after MG infection of the trachea in chickens, helping to understand how MG causes avian respiratory diseases.

Materials And Methods

2.1 Animals and bacteria

SPF chicken embryos were purchased from Jinan Sparfas Poultry Co., Ltd., and SPF chicks were self-hatched and raised by the laboratory. MG-HY was isolated, identified and stored by our laboratory^[9].

2.2 Chicken infection

Sixty 1-day-old healthy SPF chicks were randomly divided into a control group, and a test group ($n = 30/\text{group}$). Then placed in a sterile animal isolator according to standard brooding methods. The test group ($n = 30$) were artificially inoculated with MG-HY by nasal and eyes routes on day 20 of the experiment, the infectious dose was contained in 0.2 ml of medium with 1×10^9 CCU/200 μL of MG-HY

inoculum. The control group (n = 30) were received an equal dose of normal saline intranasally by eye drop. All animals were cared for according to the International Guiding Principles for Biomedical Research Involving Animals, issued by the Council for the International Organizations of Medical Sciences. Six chicks each from the control or infected cohorts were humane sacrifice of chicks by means of blood sampling from the heart at 3, 5, 7, and 15 days post-infection (dpi) for necropsy and observation of air sacs lesion. According to Zhang's research method, the pathological changes of the air sacs were classified into grades^[10]. The fresh tracheal tissue was collected and a fresh piece was fixed in 4% paraformaldehyde, and the remaining samples were stored at - 80°C for further experimental analyses.

2.3 Detection of bacterial gene load in mucosa

For determine the bacterial load invading into the mucosal epithelium, chick tracheas were aseptically removed at 3, 5, 7 and 15 days post-infection, washed with pre-chilled phosphate buffered saline (PBS, pH 7.2) and 50 mg of tissue was weighed and homogenized under aseptic conditions. DNA copy number of MG in chick trachea was detected by qRT-PCR using RocheLightCycler instrument (Roche, Shanghai, China). MG specific primers MG16sRF (5'-CGCAAGAATGAACTCAAACGG-3'; melting temperature Tm57.80°C) and MG16sRR (5'-GACACGAGCTGACGACAACCA-3'; Tm60.6°C). The DNA template was extracted from samples or elution buffer (negative control) with a bacterial DNA kit (Omega Bio-tek Inc., Norcross, Georgia, USA). The in-vitro standard DNA curve was plotted by the numbers of MG derived from the culture method and cycle threshold (Ct) values obtained using qRT-PCR^[11].

2.4 Histopathological examination

Fresh tissues were removed and fixed in 4% paraformaldehyde for 36 h. Dehydrated in graded ethanol, and then embedded in paraffin wax. The tissues were sliced at 3 µm thick and then stained with hematoxylin and eosin (H&E). The scoring was mainly based on the presence or absence of shedding and hyperplasia of tracheal epithelia tissue, thickening of the submucosal tissue and presence of lymphocytes. All parameters were scored separately from 0 (lesion absent) to 3 (severe lesion).

2.5 RNA extraction, library construction and sequencing

Total RNA from the tracheal tissue of the chicks was extracted using Trizol reagent (Sangon, Shanghai, Beijing) following the manufacturer's protocol. Qubit 2.0 Fluorometer and Qubit 2.0 RNA Kit (Thermo, Shanghai, China) were used to evaluate the quality and quantity of RNA, and the integrity and contamination of total RNA were detected by RNase-free agarose gel electrophoresis. Equal quantities of total RNA from each group of three animals were mixed prior to Illumina cDNA library preparation. mRNA was purified using oligo (dT) magnetic beads. By using RNA fragmentation reagent (Ambion), the mRNA was fragmented into short fragments (about 200 bp), and then the first strand of cDNA was synthesized by reverse transcription with random hexamer-primer using the mRNA fragments as templates; RNase H, and DNA polymerase I were added to synthesize the second strand. And through PCR amplification, end repair/poly(A) addition, and finally, the sequencing adapter is connected to the double-stranded cDNA. Enriched PCR products were purified using Agencourt Ampure XP Beads (Beckman Coulter) and enriched

by PCR amplification to create a cDNA library. The cDNA library was sequenced on the Illumina sequencing platform (HiSeqXTen)^[12].

2.6 Bioinformatics analysis of RNA-seq data

The quality of raw reads was assessed using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), the 3' end sequencing adapter sequences and low quality (quality less than 20 bases) bases were removed. Sequences after QG of each sample were aligned to the NCBI database Gallusgallus reference genome using tophat software^[13]. Using RseqQC software (Fig. <http://rseqc.sourceforge.net/>), assessed for RNAseq sequencing. Differentially expressed genes were identified by DESeq software, differentially expressed genes were screened using $\log_2FC > 1$ or < -1 and $FDR < 0.05$ as thresholds, all differentially expressed genes compared were combined, and the expression intensity values obtained by scatter plot-based analysis^[14]. The $\log_2$ fold changes ≥ 2.00 DEGs identified by DESeq software were analyzed, by gene ontology (GO) (Fig. <http://www.geneontology.org/>), to identify enriched biological processes. Finally, to understand the biological pathways associated with DEGs, the DEGs were annotated by the KEGG Automatic Annotation Server (KAAS) (Fig. <http://www.genome.jp/tools/kaas/>), a web-based Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis program run to obtain functional annotations^[15]. Statistics and visualization of all expression data were performed by R package software (Fig. <http://www.r-project.org/>)^[16].

2.7 Immunohistochemical analysis

The oven-dried paraffin sections were routinely deparaffinized with xylene and hydrated in an ethanol gradient, antigen was repaired with citrate buffer, cooled to room temperature, and incubated in 3% hydrogen peroxide aqueous solution for 10 min to inactivate endogenous enzymes, after rinsing with PBS for 5 min $\times$ 3 and then placed in a humidified box of 5% normal goat serum at room temperature for 30 min, the primary antibody (claudin-1, 1:100, purchased from Abcam, USA; occludin, 1:150, purchased from Boosen, China; ZO-1, 1:200, purchased from Boosen, China) was incubated overnight at 4°C, rinse with PBS for 5 min $\times$ 3, add the diluted secondary antibody and incubate in an incubator at 37 ° C for 30 min, wash, DAB color, counterstain with hematoxylin, mount with neutral gum, and observe under a microscope. PBS instead of the primary antibody was used as a negative control, and the presence of brownish-yellow cells in the tracheal tissue was used as a positive expression^[17].

2.8 FISH Assay

To determine the effect of MG infection on mucosal epithelial injury, the distribution of MG within the tracheal tissue was determined by immunofluorescence microscopy. In brief, tracheal tissue sections were deparaffinized, digested in xylene, soaked in denaturation buffer at 78°C for 8 min, and then 20 μ L hybridization buffer containing 15 μ g of probe was added to each section to mix well and hybridized overnight at 37°C. In this study, MG was detected and labeled with Cy3 using the hybridization probe LmCq, 5'-TGCGAATGTACTCAGGCAGGATGTTTAATGTG (ttt CATCATCAT ACATCATCAT). After washing with washing buffer (50% formamide, 50% 2 $\times$ SSC) for 5 min $\times$ 3 times, cell morphology was determined

by counterstaining with DAPI (4', 6-diamidino-2-phenylindole) staining solution for 10 min, and images were observed and collected using a fluorescence microscope (Nikon).

2.9 Quantitative Real-Time RT-PCR (qRT-PCR)

To further validate the reliability of transcriptome data, the relevant genes involved in the activation of ERK-MLCK signal transduction pathway (TNF- α , Ras, RafB, ERK1/2, c-fos and MLC2) were selected for detection by qRT-PCR based on the transcriptome sequencing results. β -actin was used as a reference gene to normalize the transcription level of the target gene. All measurements were performed according to the manufacturer's instructions, with three replicates for each sample. PrimeScript™ RT Master Mix kit (TaKaRa, Dalian, China) was used to reverse-transcribe total RNA into cDNA, and gene-specific primers were used for qRT-PCR amplification. The primer sequences are in Supplementary Table 1. The qRT-PCR reaction program was as follows: initial denaturation at 95 ° C for 2 min, followed by 40 cycles of denaturation at 95 ° C for 5 s and annealing at 60 ° C for 30 s. The $2^{-\Delta\Delta Ct}$ method was used for each sample data, and relative quantitative analysis was performed relative to the expression level of β -actin in each sample [18].

3.0 Statistical Analysis

All data were expressed as means $\pm$ Standard Error of Mean (SEM). All statistical analyses were performed using GraphPad Prism 8 software. Data between groups in H&E experiments were analyzed by Mann-Whitney U tests (Prism 8.0). All the other data between two groups were evaluated using unpaired, two-tailed Student's *t*-test (Prism 6.0). Data among two groups were analyzed by the one-way ANOVA, which was used to determine the significance of differences among groups (** $p < 0.05$ and *** $p < 0.01$).

Results

4.1 MG infection causes abnormal tracheal tissue in chicks

After infection, the air sac lesions grades were analyzed. There was a significant difference between infected group and control group. After MG post-infection, the air sacs of all chicken produced different degrees of lesions post-infection, whereas, the control group were all grade 0, with normal air sacs color and no secretion (Fig. 1A). Then, the DNA copy number of MG in the trachea was detected via qRT-PCR, the copy number of infected chicks showed an increasing trend with the extension of infection time (Fig. 1B), while MG was not be detected in the control group. The trachea lesions in chicks were explored by H&E staining. In chicks exposed to MG, there were more severe lesions (Fig. 1C). Different degrees of detachment of tracheal epithelial tissue, subepithelial infiltration of inflammatory cells (Fig. 1C-2), and thickening and hyperplasia of submucosal tissue (Fig. 1C-3) were observed in the infected group, while no significant histological abnormalities were observed in the control chickens (Fig. [link](#) [rid="fig1">1</link>C-1](#)).

4.2 Evaluation of Transcriptomic Sequencing Quality

The transcriptome data derived from the trachea cDNA libraries prepared from the infected and control samples produced more than 55 million original readings, respectively. After filtering out low-quality bases, 57745164 and 54132380 reads were generated from the two groups, and the effective ratios were 98.05% and 98.35%, respectively. After mapping clean reads onto *G. gallus* to assess the quality of sequencing, and the obtained mapping read lengths of the two groups were 48771234 and 50886334, respectively, with the mapping proportions of 92.93% and 91.48%, respectively. Moreover, the percentage of GC content in all these samples was above 50%. All the assessment criteria of base quality (Q30) were more than 94%. All data about quality of transcriptomic sequencing were shown in Supplementary Table 2.

4.3 Screening and identification of DEGs

To identify the differentially expressed genes, the transcriptome data of chicks trachea from 15 days after MG-infected were analyzed by using the DESeq package and edgeR software in R software. There were total 3112 significantly DEGs ($P < 0.01$) were screened between control and infected group, including 1646 up-regulated DEGs (Fig. 2A). The scatterplot results showed that the magnitude distribution of the significantly changed genes in the above comparisons (Fig. 2B). Annotation of the differentially expressed genes was achieved through BLASTN similarity searches against the NCBI chicken RefSeq mRNA database. The complete list of DEGs were shown in Supplementary Table 3.

4.4 GO and KEGG Pathway Enrichment Analyses of DEGs

To perform overview of Gene Expression Profiles for Transcriptome Libraries of the differentially expressed genes identified by DESeq analysis, GO analysis of differentially expressed genes was carried out by DAVID. Total DEGs were annotated by 11377 GO terms. Up-regulated DEGs were significantly ($p < 0.05$) annotated to 974 GO terms while down-regulated DEGs were significantly ($p < 0.05$) annotated to 1238 GO terms. GO enrichments were divided into biological process (1986), cellular component (2030) and molecular function (1855) (Table 1). Because some genes are grouped into more than one subcategory, the sum of genes in these subcategories may exceed 100% (Supplementary Tables 4,5).

Fifteen top GO enrichments in each functional ontology were showed in Fig. 3. In up-regulated DEGs, the dominant GO terms of biological process were “GO:0050896 response to stimulus” and “GO: 0032501 multicellular organismal process”; of cellular component were “GO:0005856 cytoskeleton” and “GO:0044430 cytoskeletal part”; and of molecular function were “GO:0003824 catalytic activity” and “GO:0005515 protein binding” (Fig. 3A). In down-regulated DEGs, they were “GO:0065007 biological regulation” and “GO:0050789 regulation of biological process” in biological process; “GO:0016020 membrane” and “GO:0044425 membrane part” in cellular component; “GO:0005515 protein binding” and “GO:0043167 ion binding” in molecular function (Fig. 3B).

To identify the biological pathways that are active in the chicks trachea at the early stages of MG-infected, 3112 differentially expressed genes were mapped to canonical signaling pathways found in KEGG. The criteria of p -value < 0.05 was chosen to determine the pathway enrichment of significantly up-regulated or down-regulated genes (supplementary table 6,7). The KEGG pathway enrichment analysis

demonstrated that most up-regulated DEGs were associated with immune defense response, including “ko04514 : Cell adhesion molecules (CAMs),” “ko04145 : Phagosome,” “ko04022 : MAPK signaling pathway,” “ko04020 : Calcium signaling pathway,” “ko03320 : PPAR signaling pathway” (Fig. 4A). Additionally, the most enriched pathways among down-regulated DEGs were associated with respiratory mucosal epithelial cell migration and injury including “ko04530 : Tight junction,” “ko04512 : ECM – receptor interaction,” “ko04510 : Focal adhesion,” “ko04152 : AMPK signaling pathway,” “ko04115 : p53 signaling pathway,” “ko04015 : Rap1 signaling pathway,” and “ko04810 : Regulation of actin cytoskeleton” (Fig. 4B).

4.5 Signal transduction pathway of mucosal injury induced by MG infection

GO classification (Fig. 3 and Additional file 4,5) indicated that related genes involved in immune defense and mechanical barrier of tracheal mucosa were enriched, specifically under GO terms “response to stimulus,” “biological regulation,” and “cytoskeleton”. As an important component of the tracheal mucosal barrier, the mechanical barrier is mainly composed of TJ junctions between tracheal epithelial cells and adjacent cells. Previous studies have shown that MG infection can activate local immunity in the tracheal mucosa, cause increased levels of pro-inflammatory cytokines, and mediate cytokine imbalance^[19]. Inflammatory cytokines (TNF- α) can activate multiple signal transduction pathways to regulate tight junctions in tracheal epithelial cells, down-regulate the expression of tight junction-related proteins, and cause increased permeability of tracheal mucosa, such as myosin light chain kinase (MLCK), protein kinase C (PKC), protein kinase A (PKA), Rho and mitogen-activated protein kinase (MAPK), among which MAPK signal transduction pathway plays an extremely important role in regulating the expression of TJ proteins^[20]. As shown in Fig. 5, Ras (Fig. 5A), PKC, RafB, MEK1/2, ERK1/2, and MLCK were up-regulated and tight junction proteins claudin-1, occludin, ZO-1, and JAM4 were down-regulated in the MG infected group compared with the control group.

4.6 Effect of MG infection on mucosal epithelial injury

The results of immunofluorescence microscopic examination were shown in Fig. 6. It has been examined that a large amount of red fluorescent signal (MG) was distributed in the mucosal epithelium, submucosa, and adventitial regions in the tracheal tissue of chicks at day 15 MG-infection compared to control group. The bacterial load within the tracheal tissue was significantly higher in MG-infection group compared to control group at 7- and 15-day post-infection than at 3- and 5- dpi, and at 15 dpi, the red fluorescence signal intensity rose with the greatest span and the highest bacterial load.

4.7 Experimental Verification of DEGs

To further confirm the transcriptomic sequencing, the relevant genes involved in the activation of the ERK-MLCK signal transduction pathway were selected to be detected by qRT-PCR. Figure 7 showed expression level of DEGs (TNF- α , INF- β , Ras, MEK1/2, MLCK and ERK1/2) by RNA-seq and qRT-PCR, respectively. Similar to that observed with RNA-seq analysis, they are found to significantly increase by qRT-PCR analysis (Figs. 7A,B). In addition, to further confirm the effect of ERK signal transduction pathway

activation on tight junction protein expression levels in vivo, the expression of claudin1, occludin, and ZO-1 in tracheal tissues signaling were analyzed with immunoblotting. We found that abundance of protein in the tracheal of infectious chicks were significantly lower, compared to control group (Fig. 7C). The data for these differentially expressed genes exhibited a similar expression tendency as the RNA-seq results, confirming the reliability of the transcriptome sequencing results.

Discussion

In poultry respiratory system, trachea acts as the air channel for gas exchange and composes the first line of defense against pathogenic microorganisms^[21], factors that make it a target of pathogenic attack such as MG. MG infection mainly causes tracheal mucosal epithelial injury, which destroys the permeability and stability of the mucosa, leading to the entry of a large amount of tissue fluid into the tracheal and alveolar spaces, ultimately causing pulmonary ventilation/ventilation dysfunction in chicks and the phenomenon of respiratory distress^[22, 23]. Thus, intranasal infection of MG was used in this study. The effects of MG infection on the structural integrity of the tracheal mucosal epithelium and the function of intercellular tight junctions in chickens were studied in detail.

During the animal experiment, by bacterial counting analysis the bacterial load in the trachea of chickens revealed that MG proliferated in the trachea of chicks after inoculation, and the bacterial load in the tracheal tissue samples showed an increasing trend with the extension of infection time during the 15 day post-infection, and caused air sac lesions in chickens. In addition, MG infection impaired the structural integrity of the tracheal mucosal epithelium in chickens. Histopathological examination showed that the mucosal epithelium of the infected group had different degrees of shedding, hyperplasia, and thickening of the submucosal tissue, and the infiltration of inflammatory cells led to the expansion of the lamina propria, which was consistent with the results of previous studies on MG. MohammedAetal. (2005), found that challenge of the trachea of chicks using the *Mycoplasma gallisepticum* R_{low} strain resulted in infiltration of a large number of lymphoid cells (CD4 + and CD8 +) as well as plasma cells secreted by immunoglobulins (IgA and IgG), resulting in expansion of the lamina propria^[24]. Based on these findings, it can be speculated that MG infection can cause damage to the tracheal mucosal epithelium and disrupt the normal function of the tracheal mucosal barrier. However, the underlying mechanism of host inflammatory responses to this bacterial infection is still elusive. Thus, the DEGs in the tracheal of MG- infected chickens are explored in this study with RNA-seq to understand its pathogenic mechanism.

In the RNAseq data analysis, a total of 3112 significant ($p < 0.05$) DEGs were selected between MG-infection group and control group, including 1646 up-regulated genes and 1466 down-regulated genes. From the results of the GO enrichment analysis, there were more genes in the subcategories involved in the defense response and mechanical barrier of tracheal mucosa. In parallel, cytokine and chemokine activities were enhanced. A previous study using high-throughput sequencing to study the effect of MG on the immune pathway in chicks, it was found that the expression of genes encoding proteins with

immune-related functions increased after *Mycoplasma gallisepticum* R_{low} strain infection, causing high levels of cellular stress and further promoting immune cell infiltration. In this study, the expression of various inflammatory factors, including collagen alpha-2 (XI) chain, fibrinogen alpha chain (FGA), dermatan sulfate proteoglycan (EPYC), folate receptor 1 (FOLR1), TNF- α , IL-6, IL-1 β , and CCL20, was increased in the trachea of MG-infected chicks. Collagen alpha-2 (XI) is the most abundant fibrous protein in the extracellular matrix and has a role in regulating inflammatory cell in inflammation models^[25]. Which was consistent with the results that was observed through the histochemical analysis. EPYC and FGA play a vital role in a variety of pathophysiological processes, such as cell migration and inflammation, which increased during the inflammatory response and participate in persistent inflammation^[26]. FOLR1 plays a crucial role in inflammation and its level is reported increased during the inflammatory response. TNF- α is a cytokine mainly secreted by monocytes and macrophages after activation by inflammation. By binding to its specific receptor, it regulates the metabolic activity of other tissues and impairs the function of endothelial cells^[27]. IL-6 is a multipotent cytokine with a wide range of functions, which has the function of regulating immune response, and plays an important role in the anti-infective immune response of the body^[28]. IL-1 β is an important inflammatory factor, which participates in the body's immune response, induces apoptosis and directly kills target cells, etc^[29]. CCL20 is able to control immune cell-directed chemotaxis by interacting with G protein-linked transmembrane receptors and is involved in the inflammatory pathology of many diseases. Therefore, the up-regulated pro-inflammatory factors and cytokines after exposure to MG may play a role in the pathogenesis of MG-induced tracheal mucosal epithelial injury.

Previous studies reported that invasion of the body by external pathogens activates local immunity in the tracheal mucosa, leading to the development of inflammation and disrupting the respiratory mucosal barrier^[30]. In this study, we found that several genes involved in constituting the structural basis of the mucosal barrier were down-regulated, including CRNN, claudins, ZO-1, and occludin. CRNN has been shown to play an important role in mucosal epithelial immune response and epidermal differentiation^[31]. Occludin is an integral type II transmembrane protein that plays an important role in maintaining the integrity of tight junction structures as well as the barrier function of the intestinal epithelium^[32]. Claudins, another class of transmembrane proteins involved in tight junctions discovered after occludin, constituting the major skeleton proteins of tight junctions^[33]. ZO-1, as a peripheral membrane protein, has the functions of connecting transmembrane proteins with the cytoskeleton and transmitting signaling molecules, regulating and transporting intracellular substances, maintaining the polarity of epithelial cells, and is committed to constituting a stable junctional system^[34]. These findings suggest that MG infection may lead to mucosal mechanical barrier dysfunction in chicks, decreased defense, and make chicks more susceptible to infection by other respiratory diseases.

The mechanical barrier of tracheal mucosa is composed of the connection between tracheal mucosal epithelial cells and adjacent cells, which is the most important link in tracheal mucosal immunity and can effectively prevent the passage of macromolecules, especially inhibit the route of bacterial and toxin diffusion through the tracheal mucosa into the body^[35]. MG infection stimulates the respiratory mucosal

immune system and increases the permeability of the tight junctions between tracheal mucosal epithelial cells. This is a key pathological process that causes chronic respiratory diseases in chickens^[36, 37]. From the results of KEGG pathway analysis of the two samples, it can be seen that the regulatory mechanism of tight junction proteins can explain MG-induced mechanical barrier damage in the tracheal mucosa of chicks. Lipschutz, J.H. (Fig. 2005), a survey conducted observed that MAPK signaling pathways are able to achieve modulation of tight junction-selective barrier function by up-regulating or decreasing the expression of key proteins of tight junctions^[38]. As found, stimulation of MDCK cells with cytokines (TNF α and IFN γ) significantly activated ERK1/2 and led to decreased expression of the tight junction proteins occludin, ZO-1, and claudin-1. In the presence of TNF α /IFN γ Treatment with ERK1/2 inhibitors enhances the expression of occludin and claudin-1 at the junction interface. The expression of TNF α was significantly up-regulated after MG infection, which activated the ERK-MLCK signal transduction pathway, and then the phosphorylation level of ERK1/2 was up-regulated, which translocated from the cytoplasm into the nucleus. The activated transcription factor c-fos then up-regulates the expression and activity of MLCK, regulates the distribution of TJ in tracheal epithelial cells and significantly down-regulates the major structural proteins Claudin-1, Occludin, ZO-1 and JAM4. The process may be the mechanism by which MG infection damages the tracheal mucosal epithelium.

Also, FISH with specific probe of LmCq showed a large amount of red fluorescent signals presence in the chicken embryo tracheal ring tissue. LmCq was detected widely in the mucosal epithelium, submucosa, and adventitial regions. This indicates that MG infection reduces the selective permeability of tracheal mucosal epithelium and its barrier function, provides a channel for harmful substances such as bacteria, histamine and endotoxin to enter the tissue, forms a cascade amplification effect, which further strengthens the damage to the tracheal mucosa.

To further confirm whether the transcriptome data in this study is reliable, and to explore the effects of MG infection on the mechanical barrier function of chicken tracheal mucosal epithelium, we used qRT-PCR to detect the activation of ERK-MLCK signaling pathway in tracheal epithelial cells by MG infection, and immunohistochemical analysis to assess the levels of claudin-1, occludin, and ZO-1 in each group. Results showed that the expression of related genes in the ERK-MCLK signaling pathway was significantly increased after MG infection of the trachea in chickens. And the levels of tight junction proteins (claudin-1, occludin, ZO-1) in vivo was significantly reduced after activation of the ERK-MCLK signaling pathway. The results were consistent with the transcriptome data obtained. Ras, as an upstream protein of the ERK-MLCK signaling pathway, is activated by cytokines (TNF- α), which increases the level of ERK1/2 phosphorylation and activates MLCK. The activated MLCK can cause phosphorylation of MLC, which is closely related to the increase of permeability of epithelial cells. Therefore, we speculated that MG infection would induce the secretion of cytokines (TNF- α) to activate the ERK-MCLK signaling pathway, which is the cause of tracheal mucosal epithelial injury in MG-infected chicks. Our results suggest that the regulation of TJ protein in tracheal epithelial cells by TNF- α -ERK-MLCK signal transduction pathway after MG infection caused damage to the chicken tracheal mucosal

epithelium. Nevertheless, the underlying mechanism of tight junction protein regulation induced by *Mycoplasma gallisepticum* infection need to be further studied.

Abbreviations

MG $\square$ Mycoplasma gallisepticum

CRD: Chronic respiratory disease

RNA-seq: Transcriptome sequencing

DEGs: Differentially expressed genes

qRT-PCR: Quantitative real-time PCR

FISH $\square$ Fluorescence in situ hybridization

SFP: Specific pathogen free

ERK $\square$ Extracellular-signal regulated protein kinase

MLCK $\square$ Myosin light chain kinase

MLC $\square$ Myosin light chain

TJ $\square$ Tight junction

TNF- α $\square$ Tumor necrosis factor-alpha

IFN- γ : Interferon gamma

CCL20: Interleukin 20

Dpi: Days post infection

PBS: phosphate buffer saline

H $\square$ E: Hematoxylin and eosin

QG: Quality control

log₂FC: fold-change

FDR $\square$ P value corrected

GO: Gene ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

KAAS: KEGG Automatic Annotation Server

DAPI 4,6-diamino-2-phenyl indole

DAB Diaminobenzidine

ERK1/2 Extracellular signal-regulated kinase

MLC2 Myosin light chain 2

SEM Standard Error of Mean

Q30 Base quality 30

TPM Transcripts Per Million

CAMs Cell adhesion molecules

AMPK AMP-activated protein kinase

PKC Protein kinase C

PKA Protein kinase A

MAPK Mitogen-activated protein kinase

FGA: Fibrinogen alpha chain

EPYC: Dermatan sulfate proteoglycan

FOLR1: Folate receptor 1

IL-1 β : Interleukin 1 beta

Declarations

Ethics approval

The animal protocols used in this work and all experimental procedures were performed in compliance with the laws and guidelines of the Fujian Agricultural and Forestry University Animal Care and Use Committee (Approval No: PZCASFAFU201904).

Conflict of interest statement

The authors declare no conflict of interest. The funders had no role in the study design, data collection, analysis, and interpretation, the writing of the manuscript, or the decision to publish the results.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files.

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Table 1 Functional Classification of DEGs

Group	Significantly differential gene			All Genes
	Up	Total	Down	
biological process	991	1986	995	12647
molecular function	920	1855	935	11860
cellular component	1036	2030	994	13059

Figures

Figure 1

Chicken model of chronic respiratory disease caused by MG. (A) Grades of air sac lesions in chicks caused by MG (n = 6, graphpadprism8 analysis). (B) Load analysis of bacteria in the trachea, the copy number of MG strains was determined by qRT-PCR after bacterial infection. (C) Score of severe tracheitis lesions caused by MG with HampE staining. (D) Micrographs of H&E staining of trachea from MG

infected control chickens (n = 6, magnification 400 ×). Scale bar, 50 μm. (1) In the control group, the trachea was intact without thickening. (2) Shedding of tracheal epithelial tissue (upper arrow of lesion) and inflammatory infiltration (lower arrow of lesion) in the infected group. (3) Hyperplasia of tracheal epithelium in infected group. Data are presented as mean ± SEM. *P < 0.05, ***P < 0.001.

Figure 2

Scatter plot of differentially expressed genes between MG infected group and control group. (A) Histogram of differentially expressed genes in MG infected group and control group. (B) Scatter plot of differentially expressed genes: The log (TPM) values of the samples with two expressions on the X and Y axes were presented, with red dots (Up) and green dots (down) indicating significant gene differences (P < 0.01), and black dots indicating non-differential genes.

Figure 3

GO enrichment analysis of DEGs in trachea of MG-treated chickens. (A) Shows the top 15 enriched GO terms of up-regulated DEGs and panel (B) Shows the top 15 enriched GO terms of down-regulated DEGs.

Figure 4

KEGG pathway enrichment analysis of DEGs in the trachea of chickens exposed to MG. (A) KEGG pathway enrichment analysis up-regulated the top 30 enriched pathways of DEGs. (B) The top 30 enriched pathways of down-regulated DEGs.

Figure 5

DEGs involved in tight junction protein regulatory mechanisms, red background: indicate up-regulated genes, blue background: indicate down-regulated genes; green shading represents localization to genes in the GallusGallus genome, also indicating the integrity of the pathway.

Figure 6

Immunofluorescence microscopy showed a specific distribution in tracheal tissue after MG infection and a significant rise in bacterial numbers with prolonged infection. Micrographs are presented as DAPI, Cy3 and Merge. Photomicrographs were taken at 200X magnification (n = 3).

Figure 7

Relevant genes involved in the activation of the ERK-MLCK signal transduction pathway were upregulated at 15 dpi. (A) RNA-seq revealed high expression of genes involved in the ERK-MLCK signal transduction pathway after *M. gallisepticum* infection ($n = 3$). Fold change was measured by the ratio of infected test group basemean to control group basemean after RNA-seq. (B) qRT-PCR was performed to detect the high expression of genes related to the ERK-MLCK signal transduction pathway after *M. gallisepticum* infection ($n = 6$, unpaired two-tailed t-test) (C) CLaudin-1, occludin, and ZO-1 protein expression abundance in the trachea of chicks by immunohistochemical analysis ($n = 6$, unpaired two-tailed t-test) at a magnification of 200X. All data are expressed as mean $\pm$ SEM.* $P < 0.05$, *** $P < 0.001$.

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