Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Experimental Investigation on *Ornithobacterium rhinotracheale* and *Enterococcus faecalis* Co-Infection in Chickens

Peng Zhao[1], Guojiang Wu[2], Qiang Zhang[1], Jun Chu[1], Chen Xie[1], Yifei Wang[1], Yanxin Deng[1], Yuxin Hao[1] and Cheng He[1]*

[1]Key Lab of Animal Epidemiology and Zoonosis, Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, 100193 Beijing, China; [2]College of Life Sciences, Agricultural University of Heibei, Baoding 071000, China
*Corresponding author: hecheng@cau.edu.cn

ARTICLE HISTORY (14-333)

Received: June 28, 2014
Revised: October 10, 2014
Accepted: January 05, 2015

Key words:
Chickens
Con-infection
Enterococcus faecalis
Hemorrhagic pneumonia
Ornithobacterium rhinotracheale

A B S T R A C T

A severe hemorrhagic pneumonia with respiratory distress prevalent has been observed in broilers with approximately 80% morbidity and 50% mortality. In our initial studies**,** *Ornithobacterium rhinotracheale* (ORT), *Enterococcus faecalis (E. faecalis)* and *Streptococcus zooepidemicus* were isolated from the diseased birds. However, the respective contribution of these organisms in the pathogenesis of hemorrhagic pneumonia is unclear. The objective of this study was to evaluate the role of ORT-*E. faecalis* co-infection in hemorrhagic pneumonia in chickens. Biochemical assays and 16S rRNA-based PCR were used to identify 5 *E. faecalis* isolates. Subsequently, forty-eight 21-day-old SPF chickens were divided randomly into six groups, 8 birds in each group. Chickens were co-infected intraperitoneally with ORT and *E. faecalis* isolates simultaneously, or inoculated with ORT first, then with *E. faecalis* three days later, and vice versa. Control groups consisted of chickens inoculated with ORT alone or *E. faecalis* alone. Post inoculation, the mortality was 87.5, 62.5, 37.6, 62.5 and 50% in birds co-infected with ORT and *E. faecalis*, infected with *E. faecalis* first followed by ORT, with ORT first followed by *E. faecalis*, and mono-infected with ORT and *E. faecalis*, respectively. Moreover, serum specimens from 60 out of 194 randomly selected chickens (30.9%) were positive for antibodies against *E. faecalis*. Our co-infection studies suggest that *E. faecalis* infection is able to trigger hemorrhagic pneumonia with a rapid mortality, while ORT infection was able to prolong pathological lesions. Therefore, the occurrence of ORT and *E. faecalis* co-infection may be associated with the outbreak of chicken hemorrhagic pneumonia.

To Cite This Article: Zhao P, G Wu, Q Zhang, J Chu, C Xie, Y Wang, Y Den, Y Hao and C He, 2015. Experimental investigation on *Ornithobacterium rhinotracheale* and *Enterococcus faecalis* co-infection in chickens. Pak Vet J, 35(2): 173-177.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative bacterium associated with respiratory diseases in many avian species, causing significant economic losses in the poultry industry due to growth retardation and the discarding of infected carcasses as unacceptable for human consumption. It has been isolated from many bird species, such as: pheasant, pigeon, rook, duck, ostrich, goose, guinea fowl, turkey, chicken, red-legged partridge and falcon (Canal *et al*., 2005; Chansiripornchai *et al*., 2007; Van Empel *et al*., 1996). ORT affects the respiratory tract causing severe respiratory signs, depression, reduction in feed uptake and growth rate. It can be a primary or secondary etiological agent with avian pneumovirus (APV), Newcastle disease virus (NDV), influenza virus and *Escherichia coli* (Van Empel and Hafez, 1999; Thachil *et al*., 2009). Outbreaks of respiratory disease associated with ORT have been reported all over the world, including China, Germany, England, Slovenia, Mexico, Peru, Jordan, Egypt, Canada, Brazil, Belgium, Israel, USA, France, the Netherlands, Hungary, Korea, Japan, Taiwan, Turkey, and South Africa (El-Sukhon *et al*.,2002; Erganis *et al*., 2002; Canal *et al*., 2005; Misirlioglu *et al*., 2006; Chansiripornchai *et al*., 2007; Asadpour *et al*., 2008; Hafez and Vandamme, 2011). Recent reports disclosed that co-

infection with ORT and H9N2 or *Streptococcus zooepidemicus* contributed to outbreak of chicken bronchial embolization as well as broiler airsacculitis (Pan *et al*., 2012a; Pan *et al*., 2012b).

Enterococci are members of the normal microbiota of the gastrointestinal and urogenital tracts of humans and animals (Pan *et al*., 2012a; El-Ashry *et al*., 2013). In chickens, *Enterococcus faecalis* and *Enterococcus faecium* were found to constitute the dominant bacterial flora of the intestinal tract in 1-day-old chicks, while *Enterococcus cecorum* was found to be dominant in birds older than 12 weeks. In addition, these organisms have the potential to cause clinical infections (Devriese *et al*., 1991). In poultry, *E. faecalis* has been isolated from septicemia with valvular endocarditis (Chadfield *et al*., 2004), growth depression and amyloid arthropathy (Landman *et al*., 1994), pulmonary hypertension syndrome (Tankson *et al*., 2001). *E. faecalis* also occurs as a potential pathogen in other animals, and is the most common *Enterococcus* sp. associated with infections in humans (Malani *et al*., 2002). Very little is known about the pathogenesis and epidemiology of infections in avian species due to the infection occurring in all age groups. However, the most serious infections have previously been associated with the late embryos mortalities and high death in very young birds due to the egg contamination (Wages, 2003). Recent observations on the causes of mortality in broiler parent flocks have demonstrated an increased mortality due to *E. faecalis* (Gregersen *et al*., 2010). Information on the epidemiology and lesion types associated with this organism in broiler parents, however, is almost non-existent.

In recent years, respiratory disease of unknown etiology has become prevalent in broilers and young laying hens. This disease manifests itself on the first day after hatching and lasts for more than 30 days, resulting in approximately 50-70% morbidity and 30% mortality in young chickens in China. Post-mortem, hemorrhagic pneumonia and swollen kidneys, along with hemorrhagic bronchial tracts were observed. In a pilot study, ORT, H9N2, *S. zooepidemicus* and *E. faecalis* were isolated from lung, liver and spleen from commercial birds (Pan *et al*., 2012a; Pan *et al*., 2012b). Although ORT and *E. faecalis* have been isolated and identified in many cases, reports of co-infection with the aforementioned two pathogens and their potential synergistic role in disease pathogenesis have not been recorded.

MATERIALS AND METHODS

Isolation and identification of *E. faecalis*: Lungs were aseptically obtained from diseased chickens with severe pneumonia. Streak cultures were performed using standard I nutrient agar with 5% sheep blood and incubated at 37°C under aerobic conditions for 24 h. The suspected colonies were identified by Gram stain and biochemical assays (Chadfield *et al*., 2004; Pan *et al*., 2012a). DNA samples were extracted from the suspected isolates using the DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. *E. faecalis* reference strain (#C55614) originated from cattle was purchased from China Institute of Veterinary Drug Control (IVDC), Beijing. PCR was used to amplify a DNA fragment of the 16S rRNA gene of *E. faecalis*. The amplification was performed using a pair of specific primers (Francois *et al*., 2004). A 197-bp fragment was amplified and subjected to electrophoresis in a 1% (w/v) agarose gel. In this report, *E. faecalis* and ORT taxonomic designations are provided as *E. faecalis*/ species/location/time.

Determination of the LD_{50} of ORT and *E. faecalis* isolate: The current study was approved by the Animal welfare and Use Committee of China Agricultural University. The LD50 of ORT/broiler/Shandong/2011 isolates was determined as described previously (Pan *et al*., 2012b). Then, forty eight 21-day-old SPF chickens were randomly divided into six groups with 8 chickens per group. The chickens were infected intraperitoneally with different dilutions of *E. faecalis* in 0.5 ml. The inoculates of *E. faecalis* contained 5.6×10^9, 5.6×10^8, 5.6×10^7, 5.6×10^6 and 5.6×10^5 CFUs/ml. Chickens were inoculated intraperitoneally with sterile PBS as a control group. Each group was observed daily for 14 days, and the LD_{50} was determined using the Reed-Muench method (Thakur and Fezio, 1981).

Experimental infection of SPF chickens with ORT/ broiler/Shandong/2011 and *E. faecalis*/broiler/Hebei/ 2011: Forty-eight 21-day-old healthy SPF chickens were randomly divided into six groups with eight birds in each group. All birds were kept in positive pressure isolators and infected intraperitoneally with LD_{50} of the isolates in 0.5 ml PBS buffer reagents as shown in Table 2. Each group was observed daily and sacrificed on day 14 post inoculation (p.i.). The chickens were euthanized by intraperitoneal injection of sodium pentobarbital. Gross lesions of both the experimentally infected dead chickens and live birds 14 days p.i. were inspected, and the lungs of the survived chickens were collected for pathogen recovery. The ORT colonies were determined by Gram staining and the candidate colonies were further identified by PCR as described previously (Van Empel and Hafez, 1999). The *E. faecalis* colonies were evaluated by Gram staining and colony morphology on the blood agar plate.

Detection of *E. faecalis* antibody: In this study, 194 serum samples were randomly collected from 204,000 chickens in Tianjin, Hebei and Jiangsu province. These included 39 serum samples out of 39,000 laying hens aged 180-280 days, 112 from 120,000 breeder broilers aged 120-450 days, and 43 from 45,000 healthy finished broilers aged 35-42 days. Serum samples were inactivated at 56ºC for 30 min and tested for the presence of *E. faecalis* antibodies using the ELISA method. Moreover, the sera from 21-day-old SPF chickens were used as negative control. Afterwards, the optical density (OD) was read at 450nm and each sample was performed in two replications. Positive antibody responses were defined as the highest dilution that gave a ratio greater than 2.1 between test serum and the negative control serum.

RESULTS

Isolation and identification of *E. faecalis*: Five *E. faecalis* strains out of 8 *Streptococcus* spp. isolates were

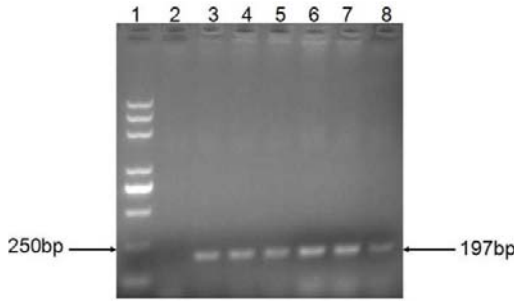

Fig. 1: PCR detection of *E. faecalis;* Lane 1: 2000bp-plus marker (Takara, Japan); Lane 2: negative control; Lane 3: Reference strain: *E. faecalis* (C55614); Lane 4: *E. faecalis*/broiler/Hebei/2011; Lane 5: *E. faecalis*/layer/Mongolia/2011; Lane 6: *E. faecalis*/broiler/Tianjin/2011; Lane 7: *E. faecalis*/Jiangsu/2011; Lane 8: *E. faecalis*/broiler/Liaoning/2011

successfully isolated from the lungs of diseased chickens by single colony purification. After 24h of anaerobic growth on the blood agar, the colonies formed by *E. faecalis* were surrounded by a pronounced zone of - hemolysis. The isolates were all Gram-positive stain under the microscope. Moreover, the isolates grew on glucose, sucrose, lactose, galactose, fructose, mannose, mannitol, and maltose, but were negative on sorbitol, arabinose A, and sodium hippurate. In contrast, the two strains could grow onto bouillon medium pH 9.6, with 6.5% NaCl, and Maconkey agar. Based on to the growth properties and analyses listed above, five strains were successfully isolated and separately named after *E. faecalis*/broiler/Hebei/2011, *E. faecalis*/layer/Mongolia/2011, *E. faecalis*/broiler/Tianjin/2011, *E. faecalis*/Jiangsu/2011 and *E. faecalis*/broiler/Liaoning/2011. The genomic DNA extracted from the five *E. faecalis* strains produced the expected 197-bp PCR product (Fig. 1).

Determination of the LD_{50} of ORT and *E. faecalis* isolate: The LD_{50} of ORT/chicken/ Shandong/2011 was determined to be 1.43×10^{8} CFUs/ml. After growth in liquid medium for 24h at 37°C, the concentration of *E. faecalis* was 5.6×10^{9} CFUs/ml. In the chicken experiment, immediate mortality occurred in group 1 after inoculation with the highest concentration. The LD_{50} of *E. faecalis* /broiler/Hebei/2011 was determined to be $5.6\times10^{7.5}$ CFUs/ml (Table 1) in SPF chickens.

Experimental infection of SPF chickens with ORT/chicken/Shandong/2011 and *E. faecalis*/broiler/Hebei/2011: One day post simultaneous inoculation with ORT and *E. faecalis*, 5 chickens out of 8 inoculated birds displayed ruffled feathers, inactivity, poor appetite and respiratory distress. More importantly, significant mortality was observed up to 7 days p.i., reaching a maximum on day 3 p.i. Total mortality amounted to 87.5% of the birds. Moreover, 3 birds inoculated with ORT first and *E. faecalis* 2 days later (ORT/*E. faecalis*) displayed clinical signs and the mortality increased gradually 3 days later while birds infected with *E. faecalis* first and ORT 3 days later (*E. faecalis*/ORT) died swiftly post inoculation with *E. faecalis* with total mortality reaching 62.5% (Table 2). In contrast, 62.5% and 50% mortality was observed in the birds infected with ORT alone and *E. faecalis* alone, respectively. Clinically, a dead peak was observed in chickens with *E.*

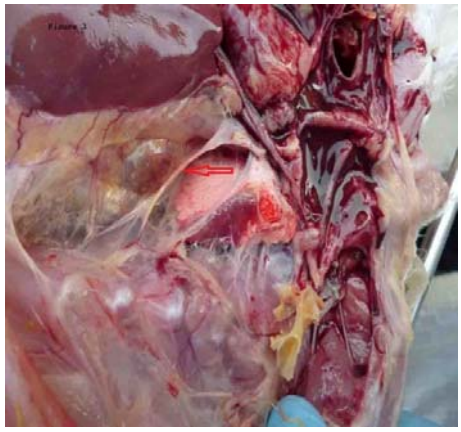

Fig. 2: At necropsy, hemorrhagic pneumonia and air sacculitis (arrow) were observed in the chickens co-infected with ORT and *E. faecalis* at same time.

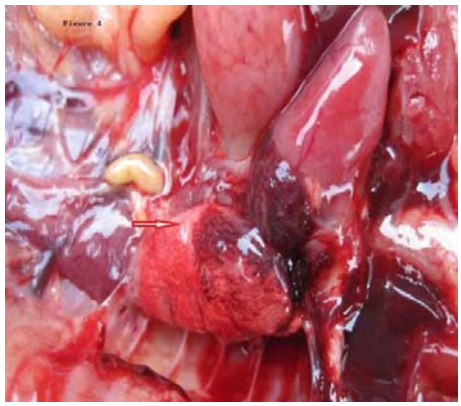

Fig. 3: Post inoculation with *E. faecalis* alone, a characteristic hemorrhagic pneumonia (arrow) was found in the dead chickens on day 5.

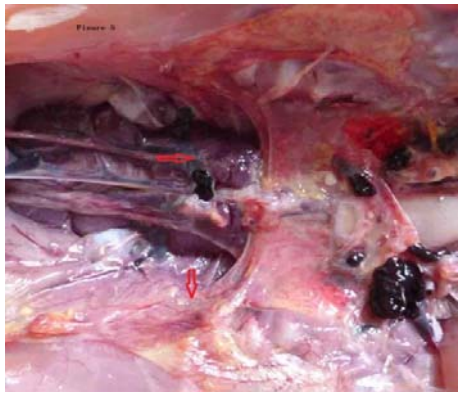

Fig. 4 Post inoculation with ORT alone, airsacculitis (arrow) and hemorrhagic nephritis (arrow) were observed in the chickens on day 6.

faecalis infection alone within 2 days and chickens were recovered 3 days later. In comparison with *E. faecalis* infection, mortality occurred 3 days later and lasted for more than 7 days.

At necropsy, hemorrhagic pneumonia, airsacculitis and hemorrhagic trachea were characteristic of chickens co-infected with ORT and *E. faecalis* at same time (Fig. 2) and in the ORT/*E. faecalis* group in contrast with the *E. faecalis*/ORT group. Moreover, chickens inoculated with *E. faecalis* alone were observed to produce a thick yellow caseous exudate, widely frothy exudates in lungs

Table 1: Determination of the LD_{50} of *E. faecalis*/ broiler/Hebei/2011 in SPF chickens

Group	No.	Dosage	Concentration	No. mortality	Mortality (%)
1	8	0.5	5.6×10^{9} CFUs/ml	7	7/8 (87.5)
2	8	0.5	5.6×10^{8} CFUs/ml	5	5/8 (62.5)
3	8	0.5	5.6×10^{7} CFUs/ml	3	3/8 (37.5)
4	8	0.5	5.6×10^{6} CFUs/ml	1	1/8 (12.5)
5	8	0.5	5.6×10^{5} CFUs/ml	0	0/8 (0.0)
6	8	0.5	PBS control	0	0/8 (0.0)

The LD_{50} was determined to be $5.6\times10^{7.5}$ CFUs/ml in SPF chickens using the Reed-Muench method.

Table 2: Experimental infection of SPF chickens with ORT/chicken/Shandong/2011 and *E. faecalis* /broiler/Hebei/2011

Groups[A]	No.	No. mortality	Mortality rate (%)	ORT recovery	*E. faecalis* recovery
1	8	5	62.5	3/3	3/3
2	8	4	50.0	0/4	3/4
3	8	7	87.5	1/1	1/1
4	8	3	37.5	3/5	5/5
5	8	5	62.5	3/3	2/3
6	8	0	0	0/8	0/8

[A] All birds were inoculated intraperitoneally. Group 1 received ORT alone while Group 2 was given with *E. faecalis* alone. Group 3 was simultaneously inoculated with ORT and *E. faecalis*. Group 4 received ORT, then received *E. faecalis* at days 3 p.i. Group 5 was inoculated with *E. faecalis*, then received ORT at days 3 p.i. Group 6 received a sterile PBS.

Table 3: Seroprevalences in broilers and layer hens flock using the *E. faecalis* antigen-based ELISA kit

Species	Ages (days)	Tested samples	No. positive	Positive (%)
Layer hens	180-280	39	8	20.5[A]
Breeder broilers	120-450	112	30	26.8[A]
Finished broilers	35-42	43	22	51.2[B]
Total		194	60	30.9

[A-B] When compared the seropositive rate between finished broilers and breeder broilers or layer hens, there was significant difference (P=0.004 or 0.003). [A-A] When compared the seropositive rate between layer hens and breeder broilers, no significant difference was found (P=0.437).

(Fig. 3) and hemorrhagic lesions in kidneys as well as bleeding in the tracheal rings. In contrast, typical hemorrhagic pneumonia and hemorrhagic nephritis were characteristic of the birds with ORT infection (Fig. 4).

Regarding pathogen recovery in the remaining birds, *E. faecalis* was isolated and identified from all co-infection groups and the *E. faecalis* alone group. However, only one ORT isolate was reisolated from the *E. faecalis*/ORT group while none was reisolated from the other groups.

Antibody detection against *E. faecalis* using ELISA method: *E. faecalis* antibodies were detected in chickens of all ages and sixty out of 194 sera (30.9%) were found positive (Table 3). With respect to species, the seroprevalence was 20.5, 26.8 and 51.2%, respectively in layer hens, breeder broilers and finished broilers. The seroprevalence was significantly higher in the finished broilers compared to laying hens and breed broilers.

DISCUSSION

In current study, five *E. faecalis* strains out of 8 *Streptococcus* spp. isolates were isolated from the lungs of chickens with hemorrhagic pneumonia. Co-infection with ORT and *E. faecalis* induced more than 87.5% mortality with hemorrhagic pneumonia and severe peritonitis in the SPF chickens in comparison with 62.5% mortality with ORT alone and 50% with *E. faecalis* infection alone. Interestingly, *E. faecalis* inoculation induced mortality rapidly while ORT infection exacerbated persistent loss. Epidemiological survey indicates that seroprevalence of *E. faecalis* is high in the three avian species. The results of this study strongly suggest that co-infection with ORT and *E. faecalis* is responsible for the current hemorrhagic pneumonia with high mortality observed in chickens in China.

Although 5 *E. faecalis* strains and 3 *S. zooepidemicus* isolates were identified from the clinical samples, the role of *S. zooepidemicus* in pathogenesis is unclear in the context of *E. faecalis* co-infection with ORT. A previous investigation revealed that the combination of ORT and *S. zooepidemicus* infection induced 100% mortality in the ORT+ *Streptococcus* group, suggesting that ORT might dominate the primary infection instead of viral infections (Pan *et al*., 2012a). In our study, *E. faecalis* infection triggered swift mortality amounting to 50% loss within 24 hours in comparison with 20% mortality induced by *S. zooepidemicus* infection (Pan *et al*., 2012a). *E. faecalis* was previously referred to be one of the most dominant bacterial species in the gut flora in 1-day-old chickens (Devriese *et al*., 1991) and it can cause endocarditis, intra-abdominal infection, pelvic infections as well as pulmonary hypertension in broilers (Tankson *et al*., 2001). Furthermore, hemorrhagic pneumonia and swollen hemorrhagic nephritis were characterized post infection with *E. faecalis* as compared to progressive bronchial obstruction in the broilers (Pan *et al*., 2012a) and bronchopneumonia in layer chickens infected with *S. zooepidemicus* (Bisgaard *et al*., 2013). Although *S. zooepidemicus* has been linked to cases of acute fatal pneumonia in dogs (Priestnall *et al*., 2010), no hemorrhagic pneumonia was recorded in young chickens. Therefore, our findings suggest that primary infection with *E. faecalis* alone may trigger hemorrhagic pneumonia and co-infection with ORT aggravates the respiratory distress and high mortality.

In this study, 8 layer hens (20.5%), 30 breeder broilers (26.8%) and 22 finished broiler (51.2%) were seropositive against *E. faecalis*. The specific antibodies were detected in 60 (30.9%) of the 194 serum samples. The results of this study indicated that the prevalence of *E. faecalis* antibodies is high in the finished broiler and breeder broilers in Northern China. Outbreaks of *E. faecalis* infection may be associated with the following factors. Firstly, excessive use of antibiotics in chickens' feed or drinking water may contribute to an overbalance of probiotics in birds' intestinal tracts, leading to excessive multiplication of *E. faecalis* and opportunistic bacterial infection in the flocks. Streptococcal and Enterococcal isolates originating from the host are regarded as commensal organisms. Concurrent enteric infections or infections via an aerosol route that compromise the intestinal villous epithelial integrity may allow penetration of resident Enterococci resulting in septicemia and endocarditis (Bisgaard *et al*., 2013). Secondly, antibiotic abuse might contribute to the infection of *E. faecalis* and ORT in poultry. In our pilot study, fosfomycin (MIC>125μg/ml), chlortetracycline (MIC>125μg/ml), spiramycin (MIC>250μg/ml),

amoxicillin (MIC>250μg/ml) and tylosin (MIC> 500 μg/ml) were detected to be multi-resistance to both *E. faecalis* and ORT isolates (Liu *et al*., 2013). Moreover, hemorrhagic nephritis induced by ORT infection immediately enables *E. faecalis* to infect other target organs via the blood stream. ORT and *E. faecalis* infections synergistically aggravate respiratory symptoms in chickens. In the present study, 87.5% mortality was observed in chickens infected simultaneously with both ORT and *E. faecalis*.

Conclusion: This study shows that co-infection with ORT and *E. faecalis* may contribute to more severe pneumonia and cause higher mortality than monoinfection with either. Above all, *E. faecalis* may dominate in a primary infection that may be followed by a secondary infection with ORT. Hence, more in-depth studies are needed to investigate the extent and impact of *E. faecalis* and ORT co-infection. Moreover, the development of a combined vaccine targeting both pathogens should be prioritized for the benefit of the poultry industry.

Authors' contribution: PZ contributed to the study and drafted the manuscript. GW performed the statistical analyses. QZ performed isolation. CX YW YD YH collected the samples. JC did ELISA test. CH contributed to the study design and obtained the funding. All authors have read and approved the final manuscript.

Acknowledgment: This work was supported by the University Research Project for college students (URP). The authors are grateful for the tremendous assistance from local veterinarians and poultry farmers with regard to obtaining samples. Also, we gratefully acknowledge the revising manuscript by Professor Patrik M Bavoil at University of Maryland, USA.

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