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Bacteriology

High prevalence of *Mycoplasma equirhinis* in Thoroughbred horses with respiratory symptoms in autumn 2018

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ABSTRACT. *Mycoplasma* species are often isolated from horses with respiratory symptoms; however, the pathogenicity of *Mycoplasma* is still unclear. In autumn of 2018, we encountered an increase in cases with respiratory symptoms, mainly coughing, in a group of Thoroughbred racehorses in Japan. We examined tracheal wash samples obtained from 40 of those cases. Bacteria and viruses that commonly cause respiratory symptoms were investigated, and anaerobes were detected in only 5 cases and *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) was detected in only 1 case of 40 cases with loop-mediated isothermal amplification assay. *S. zooepidemicus* and *Streptococcus pneumoniae* were isolated at a bacterial count of higher than 1.0×10^4 CFU/ml from 5 and 2 cases of 28 cases cultured, respectively. None of the viruses investigated was detected in 40 cases. *Mycoplasma equirhinis* (*M. equirhinis*) was isolated from 40.0% (16/40) of the cases, which was higher than previously reported isolation rates. The rate of *M. equirhinis* isolation in the cases from 2018 was significantly higher than the isolation rates in the other horses: clinical cases with respiratory symptoms in 2019–2020 (13.6%, 3/22) and healthy horses (13.5%, 5/37) in Japan. In this study, the isolation rate of *M. equirhinis* from horse group with cough symptoms in 2018 was high and no other common etiological agents were detected. The pathogenesis of *M. equirhinis* is still unclear, however, *M. equirhinis* might have been associated with respiratory symptoms in the Thoroughbred horse cases in 2018.

KEY WORDS: horse, mycoplasma, respiratory, Thoroughbred

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Respiratory disease, whose main symptom is coughing, is found in horses of all ages and is a major cause of poor performance [7, 15]. The causative agents of infectious respiratory disease are viruses and bacteria such as *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) [15]. Inflammatory airway disease (IAD) is a chronic respiratory disease, known to affect mainly young horses. A variety of etiological agents such as environmental conditions, infectious agents, and genetic influences are thought to contribute the development of IAD; however, the pathogenesis of IAD is still unclear [7].

Mycoplasmas, which are the smallest free-living organisms both in cellular dimensions and genome size, are common in mammalian species, and some species of Mycoplasmas can be pathogens in a wide variety of different animal hosts [28]. *Mycoplasma* species have been isolated from horses with respiratory disease [5], and among *Mycoplasma* species, *Mycoplasma equirhinis* (*M. equirhinis*) is often isolated in culture from tracheal wash samples of racehorses [19]. It has been reported that Mycoplasma infections might be associated with IAD in racehorses in the United Kingdom [30]; however, there is little information on the pathogenicity of Mycoplasmas in horses except for *Mycoplasma felis* (*M. felis*) which is reported to cause pleural inflammation experimentally in ponies [21]. Furthermore, there are few reports of Mycoplasmas isolated from horses in Japan, although it is said that Mycoplasma carriage may differ depending on country and climate [19].

In 2018, we encountered an increase of cases with respiratory symptoms in groups of Thoroughbred racehorses at two training facilities in Japan. Here, we show the isolation of Mycoplasmas and the investigation of bacteria and viruses from the tracheal wash samples of the cases in 2018. Furthermore, the isolation status of Mycoplasma, which was suggested to be related to the

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respiratory symptoms in the 2018 cases, was also investigated in other horses in Japan, and the isolation rates of Mycoplasmas were compared.

MATERIALS AND METHODS

Sampling from horses and investigation of the number of cases

Tracheal wash samples used in this study were taken painlessly or with slight pain from horses. This study was approved by Research Planning Committee of Japan Racing Association under the identification number 2018-3263-07. The practitioners participating this study had performed the owner informed consent. Number of the cases with respiratory symptoms and information on the cases was obtained from an in-house electronic medical record system.

Cases with respiratory symptoms in autumn of 2018 (Group A)

Cases in one of the two training facilities, where the number of cases presenting with cough in the autumn of 2018 was increased, were designated as Group A. The training facility contains about 100 stables and keeps an average of 2,000 Thoroughbred racehorses. All cases had been vaccinated against equine influenza within the previous 6 months. Among these cases in 2018, 40 Thoroughbred cases in 22 stables sharing a training course (Group A; 2–6 years old, 24 males and 16 females) underwent endoscopy and blood tests between 0 and 38 days after the onset of cough symptoms (Supplementary Table 1). At the time of examination, all of 40 cases had coughing symptoms, 7 of the 40 cases had pyrexia (38.7–40.3°C) and 7 of the 40 cases were hyperleukocytosis (13,200–17,100/µl) (Supplementary Table 1). Seven of the 40 cases also had nasal discharge and 13 cases were receiving antibiotic medication (Supplementary Table 1). Tracheal wash samples were collected under endoscopic examination. Briefly, endoscope was passed to level 90 cm from nose, and 60 ml of 0.9% saline solution was infused and aspirated from at level of thoracic inlet [27]. Samples were immediately sent to the laboratory; samples were stored at 4°C under anaerobic conditions until laboratory analysis.

Control groups: cases with respiratory symptoms (Group B) and healthy horses (Group C)

Tracheal wash samples were collected from newly encountered cases with sporadic respiratory symptoms from 2019 to 2020 (Group B; 22 Thoroughbred horses, 0–8 years old, 8 males, 13 females, and 1 gelding) and from healthy horses that had no symptoms from 2018 to 2020 (Group C; 37 Thoroughbred horses, 1–11 years old, 14 males, 17 females, and 6 geldings). Seventeen cases of 22 cases in Group B and 15 horses of 37 horses in Group C were sampled in the training facility, respectively. Fifteen cases of Group B were receiving antibiotic medication by the time of sampling (Supplementary Table 2). All cases except for the 0-year-old case had been vaccinated against equine influenza within the previous 6 months. One tracheal wash sample was taken from each horse and used for isolation of Mycoplasmas as described below.

Detection of selected bacteria causing pneumonia in horses with loop-mediated isothermal amplification (LAMP) assay

To examine whether infection with major bacteria causing pneumonia in horses [13, 14] was present in Group A, loop-mediated isothermal amplification (LAMP) assay was performed. To obtain sediment, 1 ml of each tracheal wash sample was centrifuged at 13,000 rpm for 2 min, and 800 µl of the supernatant was discarded. The remaining 200 µl was used for extraction of genomic DNA with InstaGene Matrix (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions. The genomic DNA thus obtained was used for the LAMP assay. Reaction mixtures were prepared by using a LoopampDNA Amplification Kit (Eiken Chemical, Tokyo, Japan) in accordance with the manufacturer's instructions with primers for *S. zooepidemicus* [13], *Bacteroides–Prevotella* group, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia* [14]. To each of these seven reaction mixtures, 1 µl of genomic DNA was added and then LAMP assays were performed separately at 65°C for 60 min. The reactions were terminated by heating the mixture at 80°C for 5 min. LAMP products were detected by observing the turbidity.

Bacterial culture of tracheal wash samples

For investigation of bacterial infection in Group A, bacterial culture was performed using tracheal wash samples. One hundred µl of tracheal wash samples of 28 cases were plated on Columbia agar plates, which were composed of BBL Columbia agar base (Becton, Dickson and Co., Franklin Lakes, NJ, USA) supplemented with 5% horse blood, and incubated at 37°C overnight in aerobic and anaerobic condition. Among the cultured colonies, three major colonies were identified using MALDI Biotyper CA 3.2 System (Bruker Japan, Yokohama, Japan) as previously reported [26]. Isolates that could not be identified by MALDI Biotyper were characterized by Gram staining and oxygen requirement. In this study, the criterion for bacterial isolation associated with respiratory symptom was defined as the isolation of *S. zooepidemicus* or *Streptococcus pneumoniae* (*S. pneumoniae*) at a bacterial count of 1.0×10^4 CFU/ml or higher, based on a previous report [16].

Detection of viruses causing respiratory symptoms in horses

For investigation of viral infection in Group A, specific PCRs were performed for viruses that cause respiratory symptoms in horses. Genomic DNA was extracted from 200 µl of supernatant of each tracheal wash sample by using InstaGene Matrix, and PCR specific for equine adenoviruses 1 and 2 [8] and equine herpesviruses 1 and 4 [17] were performed as previously reported. Viral RNA was extracted from 140 µl of supernatant of each tracheal wash sample by using a Viral RNA Mini kit (Qiagen, Venlo,

Netherlands) in accordance with the manufacturer's instructions, and specific PCRs were performed as previously reported for equine Getah virus [29], equine influenza viruses [20] and equine rhinitis viruses A and B [18]. Primers used for investigation of viral infection are shown in Supplementary Table 3.

Isolation and identification of Mycoplasmas

For isolation of Mycoplasmas in Group A, B and C, 300 µl of each tracheal wash sample was cultured in NK broth base (Kanto-Kagaku, Tokyo, Japan) and incubated for 72 hr at 37°C. After incubation, 10 µl of the broth was streaked onto an NK agar base plate (Kanto-Kagaku) and incubated at 37°C under microaerobic conditions. Plates were checked daily for growth and suspected colonies were subcultured on fresh NK agar base plates. If no growth was seen after 7 days from streaking on the agar base plates, the samples were considered as negative.

Identification of isolated Mycoplasmas was performed with species-specific PCR or 16S rRNA sequencing. Genomic DNA was extracted from subcultured colonies by using InstaGene Matrix in accordance with the manufacturer's instructions. To detect *M. equirhinis* which is often isolate from horses, *M. equirhinis*-specific PCR with primers 5′-CACCGCCCGTCACACCA-3′ and 5′-GATCTCTCAAAACTGAATACG-3′ was performed for each isolate as previously described [24]. For isolates which were not able to be identified with species-specific PCR, 16S rRNA sequencing was performed in accordance with published methods by sequencing 1,500 bp PCR products amplified with primers 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1525r, 5′-AAGGAGGTGATCCAGCC-3′ [10, 11]. The obtained sequences were compared with published 16S rRNA gene sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [2].

Statistical analysis

To compare the rates of *M. equirhinis* isolation between Group A, B and C, Fisher's exact test was carried out. When significant differences were determined by using Fisher's exact test ($P<0.05$), multiple pairwise comparisons were performed to determine P values that were adjusted according to the Benjamini–Hochberg false discovery rate procedure. All analyses were performed with the R statistical package (version 4.0.4; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Number of the cases with cough symptoms in two training facilities

An increase of the cases with respiratory symptoms was observed at 2 training facilities for racehorses in autumn 2018. In the first training facility, the average number of horses with symptoms of coughing was 26 ± 8 in September and 35 ± 7 in October from 2011 to 2017. In 2018, however, 47 and 139 horses showed symptoms of coughing in September and October, respectively (Supplementary Fig. 1). In another training facility, which is geographically distant from the first training facility and belongs to same organization, the average number of horses with symptoms of coughing was 20 ± 6 in September and 29 ± 8 in October at from 2011 to 2017, however, 48 and 51 horses showed symptoms of coughing in September and October in 2018, respectively

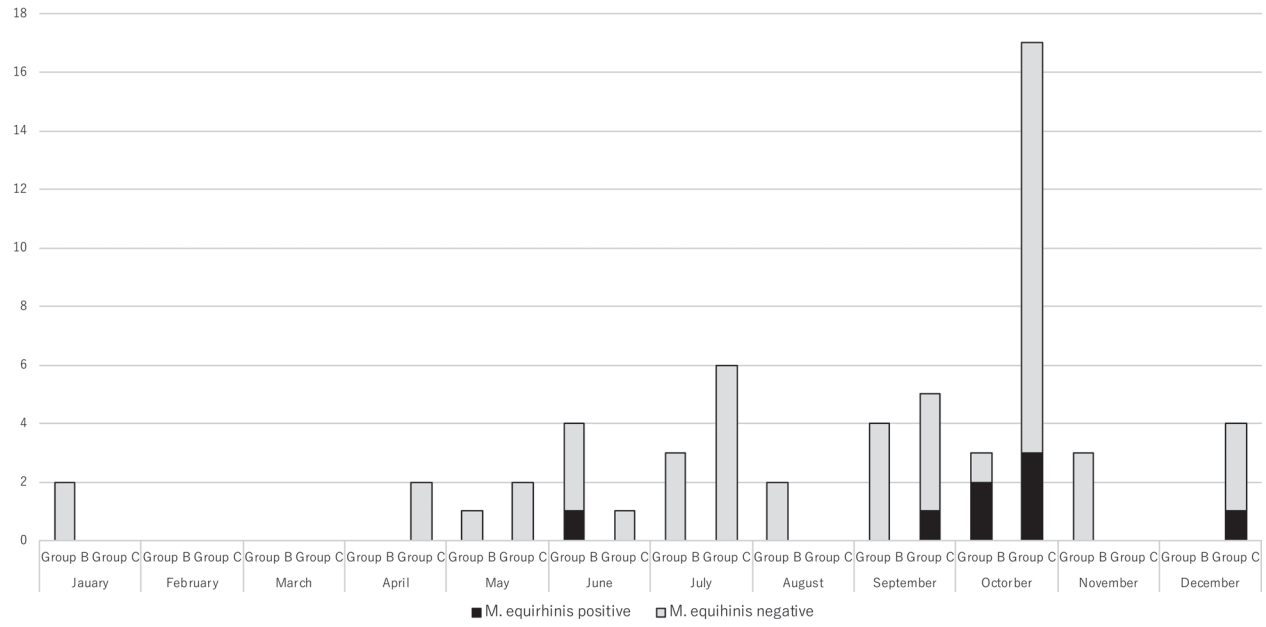

Fig. 1. Number of *Mycoplasma equirhinis*-positive (black) and -negative (gray) samples collected in each month in Group B (respiratory symptoms in 2019–2020) and Group C (no symptoms).

(Supplementary Fig. 2). Only the examination results from the first training facility (Supplementary Fig. 1) are presented in this study.

Detection of bacteria and virus in cases in autumn of 2018 (Group A)

Among the 40 cases with cough in autumn of 2018 (Group A), bacteria of the *Bacteroides–Prevotella* group was detected in 5 cases, and one of these 5 cases also tested positive for *S. zooepidemicus* (Table 1, Supplementary Table 4) with LAMP assay. The results of LAMP assays were all negative in the remaining 35 cases. Bacterial culture showed that more than one type of bacterial species was isolated from all the 28 tracheal wash samples. Up to three bacterial species isolated from each sample with the largest numbers of bacterial counts were shown in Supplementary Table 4. *S. zooepidemicus* and *S. pneumoniae* were isolated at a bacterial count of 1.0×10^4 CFU/ml or higher from 5 (A14, A17, A26, A27 and A28) and 2 (A21 and A22) samples of all the 28 tracheal wash samples cultured, respectively. None of the viruses investigated was detected in any of the 40 samples in Group A.

Isolation of Mycoplasma in cases in autumn of 2018 (Group A)

M. equirhinis was isolated from 16 cases (40.0%) (Table 1, Supplementary Table 1) and no other Mycoplasmas were detected. Among the *M. equirhinis*-positive cases, bacteria of the *Bacteroides–Prevotella* group were detected in 3 cases.

Table 1. Numbers of cases in Group A (group with respiratory symptoms in autumn of 2018) in which bacteria were detected with loop-mediated isothermal amplification assay or *Mycoplasma equirhinis* was isolated

Number of cases	*Bacteroides - Prevotella* group	*Streptococcus equi* subsp. *zooepidemicus*	*Mycoplasma equirhinis*
13	-	-	+
3	+	-	+
1	+	-	-
1	+	+	-
22	-	-	-
Number of positive cases	5	1	16

Table 2. Isolation of *Mycoplasma equirhinis* from tracheal wash samples in each group

	Number of samples	*M. equirhinis* positive	*M. equirhinis* negative	%*
Group A (with respiratory symptoms in 2018)	40	16	24	40.0†§
Group B (with respiratory symptoms in 2019–2020)	22	3	19	13.6§§
Group C (with no symptoms)	37	5	32	13.5††
Total	99	24	75	24.2

*Percentage of *M. equirhinis*-positive samples in each group. $P<0.05$ for comparison between † and ††, $P<0.1$ for comparison between § and §§.

Isolation of Mycoplasma in other horse groups (Groups B and C)

In Group B, *M. equirhinis* was isolated from 3 of 22 cases (13.6%) and 3 of 17 cases (17.6%) at the training facility (Table 2). In Group C, *M. equirhinis* was isolated from 5 of 37 horses (13.5%) and 2 of 15 (13.3%) horses at the training facility (Table 2). *M. felis* was isolated from 1 case in Group B. The percentage of *M. equirhinis*-positive samples tended to be larger in Group A than in Group B ($P=0.07$), and was significantly larger than in Group C ($P=0.03$). The percentage of *M. equirhinis* isolation was not significantly different between Group B and Group C.

Of the samples from which *M. equirhinis* was isolated in Group B and Group C, 2 in Group B and 3 in Group C were sampled in October (Fig. 1). The remaining *M. equirhinis*-positive specimens were collected in June in Group B and one each in September and December in Group C. Among 32 samples collected from the training facility in Group B and C, *M. equirhinis* was isolated from 3 samples in October, and the remaining *M. equirhinis*-positive samples were one sample each in June and December.

DISCUSSION

In mycoplasmas such as *Mycoplasma pneumoniae* pathogenic for human and *Mycoplasma mycoides* subsp. *mycoides* pathogenic for cow, it has been shown that the effects on the host immune system and host cells can lead to respiratory symptoms [22, 23]. There have been several reports on Mycoplasmas and respiratory symptoms in horses [19, 30], and an outbreak of respiratory symptoms in horses associated with *M. felis* was reported previously [31]. However, the pathogenicity of Mycoplasmas, especially *M. equirhinis*, in horses is still unclear [3, 5].

In our Group A cases, *M. equirhinis* was isolated frequently, and no other common bacteria were detected except for *S. zooepidemicus* with isolation. No viruses known to cause respiratory infection in horses were detected. The horses in Group A were kept in multiple stables, and food, bedding, or dust, were not considered to be allergens causing their respiratory symptoms. It was impossible to compare the numbers of *M. equirhinis* present in each sample because selective enrichment culture was performed as a first step in the isolation procedure used in this study. Even though the above limitation is of concern in this study, *M. equirhinis* was isolated at the highest rate among the bacteria and viruses that we examined and was the most likely to be related to the respiratory symptoms in Group A, suggesting an association with outbreaks of respiratory symptoms in Thoroughbred horses. On the other hand, 3 of the 5 cases in which *Bacteroides–Prevotella* group bacteria were detected were also *M. equirhinis*-positive. Infection with other bacteria, such as anaerobes, might have been involved in the infection of *M. equirhinis*. *S. zooepidemicus* was detected in 1 out of 40 cases by LAMP assay and 5 out of 28 cases with bacterial culture, and *S. zooepidemicus* was detected with

also LAMP assay and culture in one (A28) of them. Previous report showed that bacteria have been isolated from tracheal wash samples of horses without inflammatory findings at an average count of 1.49×10^4 CFU/ml [16], which has been suggested that bacteria are physiologically present in the tracheal wash samples of horses. Among bacteria in tracheal wash samples in horses, *S. zooepidemicus* is thought to be part of the normal bacterial microflora of the upper respiratory tract of horses [25]. However, some previous reports [16, 30] suggested that *S. zooepidemicus* or *S. pneumoniae* might be associate with airway inflammation in horses. Therefore, *S. zooepidemicus* and *S. pneumoniae* isolated at a bacterial count of 1.0×10^4 CFU/ml or higher were thought to be associate with respiratory symptoms in horses in this study. In our cases, large number of *S. zooepidemicus* was isolated from 5 cases and large number of *S. pneumoniae* was isolated from two cases in Group A, which suggested that the *S. zooepidemicus* and *S. pneumoniae* might have caused respiratory symptoms of the 7 cases.

The presence of *Mycoplasma* species in the respiratory tracts of horses has been previously reported. Mycoplasmas were isolated from 8% of nasopharyngeal swabs and 10% of tracheal swabs taken from horses with acute respiratory disease in the United Kingdom [1]. *M. equirhinis* was isolated from 10.2% of tracheal wash samples from national hunt racehorses in the United Kingdom [3] and from 16.2% of tracheal wash samples from Thoroughbred horses in Turkey [19]. In our study, *M. equirhinis* was isolated from 40.0% of cases in Group A, which was higher than the rates in Group B or Group C in this study or the rates in the British and Turkish reports. Furthermore, the *M equirhinis*-isolation rate of Group A was higher than that of groups isolated at the same facility among Group B and C. The frequency of *M. equirhinis*-isolation was higher in autumn 2018, when the number of horses with respiratory symptoms increased, than those in previous reports or in other horse groups, suggesting that *M. equirhinis* might have influenced the increase in respiratory symptoms in horses in autumn 2018.

In this study, *M. equirhinis* has also been isolated from patients treated with antibiotics (A17, A23 and A40 in Supplementary Table 1, B10 and B14 in Supplementary Table 2), and all the cases were administrated cephalothin. Mycoplasmas are resistant to beta-lactam antibiotics including cephalothin [9], and cephalothin administration may have not affected isolation status of *M. equirhinis*. However, it is difficult to discuss the effect of antimicrobial administration on *M. equirhinis* isolation, because the used antibiotics and the duration of administration varied in the cases of Group A and Group B in this study. Among the antibiotics, cephalothin was the most commonly administrated in Group A cases (10 cases in 40 cases). It is known that *S. zooepidemicus* or *S. pneumoniae* are susceptible of cephalothin [6, 12]. Although *S. zooepidemicus* were isolated with large bacterial counts from some of Group A cases (A17 and A28) even under cephalothin administration, cephalothin administration may have reduced the isolation and detection rate of *Streptococcus* species in Group A cases.

In groups other than Group A, the percentage of samples from which *M. equirhinis* was isolated was around 13%, regardless of the presence or absence of symptoms. *M. equirhinis* might, therefore, be isolated in about 13% of Japanese Thoroughbred horses under normal conditions, which is comparable to reports of cases with solitary respiratory symptoms from the United Kingdom [3] and Turkey [19]. Among the samples from which *M. equirhinis* was isolated in Group B and Group C, the majority were collected in October, although the number of samples collected per month varied and was limited. In Canada, *M. equirhinis* was isolated from 59.8% of horses with acute respiratory diseases in the autumn of 1987 [4]. In this study, cases in Group A presented with respiratory symptoms in autumn in Japan, and *M. equirhinis* was isolated in many cases. It might be possible that the season has an influence on the isolation of *M. equirhinis.*

In this study, *M. equirhinis* was isolated from 40.0% of Thoroughbred horses with respiratory symptoms in autumn of 2018, when number of horses showing cough symptoms were larger than previous years, and no other common bacteria or viruses were detected. The rate of *M. equirhinis* isolation in these cases was higher than rates in previous reports or in other Japanese Thoroughbred horses under normal conditions. It remains possible that infection of unknown pathogens, the physical condition of the horses, and seasonal or climatic effects may have affected to the increased isolation rate of *M. equirhinis* in autumn 2018. *M. equirhinis* might have damaged host's immune system as previously reported [23] and caused their respiratory symptoms as a result, without playing a role directly. However, our results showed that the isolation rate of *M. equirhinis* from horse group with cough symptoms was high in autumn 2018 and other etiological agents were excluded, except for the cases where *S. zooepidemicus* and *S. pneumoniae* were isolated with large amount. The result suggested that *M. equirhinis* may be involved in respiratory symptoms, mainly coughing, in Thoroughbred horses. In this study, it was not clear whether *M. equirhinis* was the direct cause of the cough symptoms of horses, therefore, further study including experimental infection of *M. equirhinis* should be needed. This is the first report to investigate the prevalence of *M. equirhinis* in Thoroughbred horses in Japan.

CONFLICTS OF INTEREST. The authors have nothing to disclose.

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