**RESEARCH ARTICLE**

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# Determination of *Mycoplasma haemofelis* Incidence in Cats Visiting Veterinary Clinics in Kırıkkale

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**Abstract**

This study aimed to determine the presence of *Mycoplasma haemofelis* (Mhf), which is a member of the hemotropic mycoplasmas called infectious anemia of cats, known to be zoonotic, usually progresses with hemolytic anemia in cats, and can lead to the death of the cat if not treated, in the Kırıkkale province by the Polymerase Chain Reaction (PCR) method. The experimental units of the study consisted of 50 male and 50 female cats of different breeds, aged between 2-months and 10-years, presented to the Kırıkkale University, Faculty of Veterinary Medicine, Internal Medicine, Surgery and Obstetrics Clinics with different complaints during January-August 2021. Smears prepared from blood samples were examined cytologically by the Giemsa. In addition, total blood count was done and the increased blood samples were analysed by PCR for the 16S ribosomal RNA gene. According to the PCR analysis findings of the study, it was concluded that the incidence of *Mycoplasma haemofelis* in Kırıkkale is 13 %.

**Keywords:** *Mycoplasma haemofelis*, PCR, incidence, cat.

## INTRODUCTION

*Mycoplasma haemofelis* (Mhf), previously named *Haemobartonella felis*, is a gram-negative, obligate, non-culturable, epicellular mycoplasma species that causes the breakdown of red blood cells and causes hemolytic anemia called Feline Infectious Anemia in cats (Messick and Sandos, 2011).

Feline hemotropic mycoplasmas (hemoplasmas) are found worldwide, although the prevalence varies geographically. Among the species, "*Mycoplasma haemofelis*" is considered the most pathogenic, while "*Candidatus Mycoplasma haemominutum*" (CMhm) and "*Candidatus Mycoplasma turicensis*" (CMt) are considered less pathogenic (Tasker, 2010). The zoonotic feature was confirmed by molecular identification of Mhf, which had 99 % identity with feline hemoplasm in an HIV-positive immunocompromised male patient in Brazil (Pires dos Santos et al., 2008).

Numerous comprehensive studies have been conducted on the prevalence of *Mycoplasma* infections worldwide and varying rates have been reported. According to recent data, the overall prevalence of hemoplasmas in India is 9.01 %, in Russia 13.8 % and in China 4.9 % (Malangmei et al., 2021; Demkin and Kazakov, 2021; Zhang et al., 2021).

The risk of contracting hemoplasma infection from free-range, old, and male cats has been reported to be higher than the risk of contracting from young and female cats that are domesticated (Martinez-Diaz et al., 2013). It is known that viral diseases such as feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), which cause immunosuppression, increase the risk of Mhf infection (Demkin and Kazakov, 2021).

Blood-sucking vectors play a vital role in the transmission of hemotropic mycoplasmas. Aside from that when there is an aggressive interaction between cats through an activation like scratching and bite, infected animal's saliva and faeces may play a role in natural transmission. And also blood transfusion cause infection (Willi et al., 2007).

Mhf settles on the red blood cell membrane and causes fragmentation of the red blood cells. On the other hand, by adhering to the surfaces of the red blood cells, triggering the mononuclear phagocyte system, and causing extravascular destruction of red blood cells in the spleen and liver, generally causes regenerative hemolytic anemia in cats (Kewish et al., 2004).

Because of hemotropic mycoplasmosis various pathogenicity, clinical signs are seen different (Tasker, 2010). Mhf is considered to be the most pathogenic species, and it is known to cause hemolytic anemia in immunocompetent cats. It has been stated that anemia usually does not occur in CMhm and CMt infections unless there is a concurrent disease or immunosuppression (Tasker, 2010; Korman et al., 2012).

Clinical findings depend on the period of infection, the virulence of agent and severity of anemia. In patients with severe anemia, fatigue, depression, respiratory distress, hypoxia, tachycardia, tachypnea, cardiac arrhythmias are observed. Fever, abdominal pain due to hepato and splenomegaly are other symptoms. Also, pale mucosal membranes, icterus, and pigmenturia are observed. Feline hemoplasma infections can sometimes have a latent course. Mhf is also recognized as a pathogen associated with retroviruses such as FIV, FeLV (Korman et al., 2012; Aslan, 2016).

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Hyperbilirubinemia,hemoglobinemia, hemoglobinuria and erythrocyte "ghost" cells are seen due to hemolysis (Garden et al., 2019). Acute infected cats typically develop macrocytic normochromic or hypochromic hemolytic anemia characterized by an increase in reticulocyte. In chronic cases, normocytic-normochromic anemia is observed. When it progresses together with FeLV, macrocytic-hypochromic anemia occurs (Kewish et al., 2004; Korman et al., 2012; Aslan, 2016).

In Mhf infections, leukocyte counts may be normal, and also lekuocyte levels are seen remainder or downward (Tasker et al., 2009). Metabolic acidosis, hyperbilirubinemia, increased ALT and AST levels have been reported in patients (Sykes and Tasker, 2013).

Severe hypoglycemia has been reported in sheep (*Mycoplasma ovis*), rat (*Candidatus Mycoplasma haemodidelphidis*), pig (*Mycoplasma suis*), llama (*Candidatus Mycoplasma haemolamae*), calf (*Haemobartonella bovis*), and in a cat acutely infected with hemoplasmas, however, Tasker et al. (2009) stated that they did not find any signs of hypoglycemia in their study.

Hemoplasmas can be diagnosed via the determination of the presence of the causal agent on the erythrocyte surface in blood smear preparations and by PCR analysis for the 16S ribosomal RNA gene in the blood, which is considered standard (Georges et al., 2012; Alexandre de Santis et al., 2014; Hawley et al., 2018). Enzyme-linked immunosorbent tests (ELISA) based on recombinant Mhf DNAK protein have also been developed like the positive Coombs test for the presence of erythrocyte-bound antibodies and the humoral-immune response to feline hemoplasmas (Tasker et al., 2009 b; Baumann vd., 2015).

Tetracyclines and fluoroquinolones are used in the treatment of clinical hemoplasmosis (Barker and Tasker 2013). İt is recommended that the usage of doxycycline at a dose of 10 mg/kg for 28-days, and then administering marbofloxacin at a dose of 2 mg/kg for 14-days in patients whose bacteremia continues or relapses. It has been reported that enrofloxacin is at least as effective as doxycycline when used at a dose of 5-10 mg/kg for 14-days (Lappin, 2004).

Mhf also known as infectious anemia of cats and usually progresses with hemolytic anemia, in the Kırıkkale province by polymerase chain reaction (PCR) method, as well as to statistically evaluate the relationship between clinical and hematological findings of diseased cats.

## MATERIALS AND METHODS

Ethical approval was obtained from the Kırıkkale University Animal Experiments Local Ethics Committee, with decision number 57 in a meeting dated 24/10/2020 and numbered 2020/08.

The experimental units of this study consisted of a total of 100 cats, 50 females and 50 males, from different breeds, whose ages ranged between 2-months and 10-years, which were presented to the Kırıkkale University Veterinary Faculty Research and Practice Hospital and Clinics with various complaints during January and August 2021. All animals which were recorded had contact with other animals. Following the examination for external parasite infestation, general systemic clinical examinations were performed, especially the mucosa.

For total blood count (hemogram), blood smear and PCR analysis, 2 ml of blood were taken from *Vena Cephalica Antbrachia* of the cats into EDTA (Ethylenediamine tetra-acetic acid) tubes per the technique. Two smears were prepared with one drop of each sample taken. Also, total blood count was performed with a hemogram device in the clinical sciences laboratory (Abacus Junior Vet5®, Diatron, Hungary) and the remaining blood samples were stored at -20 °C per the technique for the PCR analysis.

The two smears prepared from the blood samples were stained following the Giemsa and 150 microscope fields were examined under a light microscope with a 100-objective lens by dripping immersion oil.

PCR analysis was performed in the Sekans Animal Health Laboratory. The blood taken into the anticoagulant tubes was mixed in the laboratory for nucleic acid detection and used for DNA extraction. The DNA extraction was performed using the conventional method indicated in the diagnostic kit prepared to be used for PCR detection of Mhf nucleic acid. Following that, Agarose Gel Electrophoresis was performed using the PCR products.

A distribution analysis for cats with positive PCR results for Mhf and cats with negative PCR results was done using the Pearson chi-square test; by considering gender, age and the environment in which they were raised. Hemogram parameters were evaluated statistically with the Mann-Whitney U-test (Kalaycı, 2010).

## RESULTS

Out of the 100 cats in the study, 10 had positive PCR results. 3 were positive for both PCR result and cytological examination, and only 3 cats had a positive cytological examination. PCR was not done for the 3 cats that were positive for the cytological examination, they were considered negative for Mhf and statistical analyses were performed on the 13 cats with positive PCR test and both tests.

Considering the PCR results, relevance between keeping condition (indoor-outdoor), age and genders of Mhf positive and negative cats were given in Tables 1-3. It was observed that there was no significant statistical difference between cats kept at home or outside and the incidence of Mhf infection by gender ($p>0.05$) (Tables 1 and 2).

**Table 1.** The incidence (%) of Mhf in cats kept at home and in the garden.

| | **Home** | | **Garden** | | **Total** | |
|---|---|---|---|---|---|---|
| **Housing** | n | % | n | % | n | % |
| **Mhf (-)** | 44 | 89.8 | 43 | 84.3 | 87 | 87.0 |
| **Mhf (+)** | 5 | 10.2 | 8 | 15.7 | 13 | 13.0 |
| **Total** | 49 | 100.0 | 51 | 100.0 | 100 | 100.0 |
| $\chi^2$ (1) = 0.664; p = 0.415 | | | | | | |

(-) = Negative (+) = Positive

**Table 2.** Relationship between the incidence of Mhf and gender (Number, %).

| | **Female** | | **Male** | | **Total** | |
|---|---|---|---|---|---|---|
| **Gender** | n | % | n | % | n | % |
| **Mhf (-)** | 46 | 92.0 | 41 | 82.0 | 87 | 87.0 |
| **Mhf (+)** | 4 | 8.0 | 9 | 18.0 | 13 | 13.0 |
| **Total** | 50 | 100.0 | 50 | 100.0 | 100 | 100.0 |
| $\chi^2$ (1) = 2.210; p = 0.137 | | | | | | |

(-) = Negative (+) = Positive

Considering the age distribution of Mhf positive cats, 10 % of those were under the age of one age and ages 1-5. This rate is 40.0 % for those aged five and over (Table 3). This difference observed between the age groups was

examined with the chi-square test, the smallest expected value of the crosstab was calculated as 1.30, and it was understood that the smallest expected value in two cells (33.3 % of the total cells) was less than 5. Hence, considering the Pearson chi-square value calculated by the Exact method, it can be said that the incidence of Mhf was different between the age groups ($\chi 2(2) = 7.162$; pExact = 0.041<0.05). The source of this difference was examined with the column proportions tests. According to the results obtained with Bonferroni correction to maintain the test significance level of 0.05, it was determined that the incidence of Mhf in the 5 years and older group was significantly higher than the other groups (z = -2.5769; p = 0.01<0 .05).

**Table 3.** Age-related incidence of Mhf in cats (%).

| Age group | <1 | | > 1 - <5 | | > 5 | | Total | |
|---|---|---|---|---|---|---|---|---|
| | n. | % | n | % | n. | % | n | % |
| **Mhf (-)** | 18 | 90.0 | 63 | 90.0 | 6 | 60.0 | 87 | 87.0 |
| **Mhf (+)** | 2 | 10.0[a] | 7 | 10.0[a] | 4 | 40.0[b] | 13 | 13.0 |
| **Total** | 20 | 100.0 | 70 | 100.0 | 10 | 100.0 | 100 | 100.0 |

[a, b] Different letters in the same lines are statistically significant (p<0.05) (z=-2.5769; p=0.01).

***Clinical Findings***

Out of the total 100 cats, 29 were surgical, 6 were gynocology and reproduction, and the other 65 were referred to Internal medicine clinics and all of had different complaints. During the clinical examination, no ectoparasites were found in any of the cats, except for one with flea infestation.

Breast tumor, prolapse recti, FIP, icterus, cystitis, chemosis, fungal infection, and sacroiliac separation was observed during the clinical examination in 8 of 10 patients with positive PCR and negative cytological examination. One cat came to our clinic with a request for vaccination and one also, for dressing. One of the 3 patients with positive PCR and cytological examinations was castrated, and diarrhea and FIP were observed in the other 2 cats, respectively. Gingivitis, FIP and anorexia/fever were detected in 3 cats with positive cytological examination and negative PCR test results, however, they were not included in the statistical analysis.

***Cytological Findings***

Mhf agent was detected microscopically (pink-purple color, round, chain and ring-like on the erythrocyte surfaces) in 3 of the 13 cats that were PCR-positive and in 3 of 87 cats that were PCR-negative.

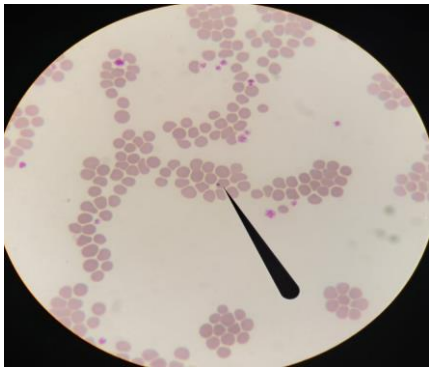

**Figure 1.** The appearance of Mhf on erythrocytes (arrow), Giemsa X 100.

***Hematological and PCR findings***

Hemogram data of Mhf positive and negative cats were shown in table 4. It was determined that there was no statistically significant difference in any of the parameters whose arithmetic mean and standard deviation were given compared to the non-parametric Mann-Whitney U test (p>0.05). The band images of the positive and negative controls and the positive samples of the PCR analysis' results on a 1% agarose gel were shown in Figure 2.

**Table 4.** Statistical analysis of hemogram parameters of Mhf positive and Mhf negative cats (Mann-Whitney U test)

| Parameter | Mean ± Standard deviation | | Mann-Whitney U | Z | P |
|---|---|---|---|---|---|
| | Mhf (-) n:87 | Mhf (+) n:13 | | | |
| **WBC $10^9$/l** | 18.53 ± 11.10 | 17.27 ± 7.64 | 553.500 | -0.123 | 0.905 |
| **LYM $10^9$/l** | 3.86 ± 2.34 | 2.77 ± 1.59 | 382.500 | -1.876 | 0.060 |
| **NEU $10^9$/l** | 13.22 ± 10.28 | 13.33 ± 6.65 | 489.000 | -0.784 | 0.440 |
| **EOS $10^9$/l** | 0.57 ± .46 | 0.40 ± .23 | 457.000 | -1.112 | 0.271 |
| **RBC $10^{12}$/l** | 7.85 ± 2.00 | 6.95 ± 3.19 | 473.500 | -0.943 | 0.351 |
| **HCT %** | 31.59 ± 7.73 | 29.49 ± 11.71 | 521.000 | -0.456 | 0.656 |
| **MCV fL** | 41.1 ± 7.1 | 46.6 ± 14.1 | 409.500 | -1.602 | 0.110 |
| **PLT $10^9$/l** | 274.6 ± 183.5 | 250.6 ± 178.0 | 525.500 | -0.410 | 0.687 |

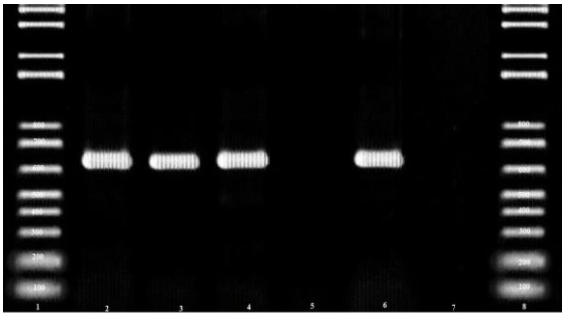

**Figure 2.** 1 % agarose gel electrophoresis image (674bp) showing 16S rRNA gene amplified products of Mhf 1-8 Ladders, 2-3-4 positive samples, 5-7 negative controls, 6 positive control.

**DISCUSSION AND CONCLUSION**

The term of hemoplasma is used to describe of the infected erythrocytes by mycoplasmas (Kewish et al., 2004). Hemotropic mycoplasmas are gram-negative bacteria that bind to the erythrocytes of mammalian hosts, can cause infectious anemia in cats, are non-producible, do not contain a cell wall (Messick and Santos, 2011).

In our study, the agents were detected on the erythrocyte surface by Giemsa staining method, in 6 out of 100 cats, in the form of a pink-purple, ring-shaped or chain form (Figure 1) (Hawley et al., 2018). Akkan et al. (2005) examined 121 Van cats with Papenheim staining method,

18 out of the 121 were positive, Atalay (2013) reported that 8 out of 84 cats were positive. Yüksel (2019) also reported that 4 out of 100 cats were positive. Gazyagci et al. (2018) found the causative agent of haemobartonella in a lion brought to Kırıkkale University Faculty of Veterinary Medicine, internal medicine clinic with complaints of nose bleeding, vomiting and anemia.

Cytological examination results for the causal agent vary widely around the world. George et al. (2012), Malangmei et al. (2021) reported that they did not find any factor in their cytological examination, while Nibblett et al. (2010) reported that they found a causative agent in 0.9 % of 115 cats and also, Akliu et al. (2016) stated that they found a causative agent in 10 % of 60 cats.

Hemoplasms have been detected in many domestic and wild animals such as cats, dogs, rodents, pigs, cattle, sheep, bears and bats. Feline haemoplasmas are found worldwide, but their prevalence can vary geographically. There are many Mhf prevalence studies conducted with the PCR in different geographies around the world. These prevalence studies include; a prevalence of 4.5 % in 262 cats in Germany, 4.8 % of 310 cats in America, 3.7 % of 191 cats in Spain, 12.81 % of 320 cats in Portugal and 5 % of 200 cats in New Zealand (Bauer et al., 2008; Sykes et al., 2008; Roura et al., 2010; Martinez-Diaz et al., 2013; Jenkins et al., 2013).

Considering recent studies, the incidence of *Mycoplasma hemofelis* is 1.3 % in a study conducted in 4880 cats in Spain and Portugal, 12 % in 111 cats in India, 5.5 % in 753 cats in Russia, 0 % in 668 cats in China, 13.6 % in 44 cats in Saudi Arabia (Mesa-Sanchez et al., 2021; Malangmei et al., 2021; Demkin and Kazokov 2021; Zhang et al., 2021; Alanazi et al., 2021).

There are limited numbers of PCR studies on the prevalence of Mhf in our country. Ural et al. (2009) reported the general prevalence of the disease as 18.9 % in their PCR study conducted on 217 cats in four different provinces (Bursa, İzmir, Ankara and Antalya). Cetinkaya et al. (2016) reported the prevalence as 9.9 % in their study conducted on 384 cats in Istanbul. In a study conducted on 100 cats in Aydın, they did not find any causative agent in PCR (Yüksel, 2019). In our study, the prevalence was found to be 13 % in cats with positive PCR tests for Mhf in the Kırıkkale province. Although, Kırıkkale and Kayseri are in the same geographical region, Atalay (2013) reported a prevalence of 4.76 % in their PCR study they conducted on 84 cats in Kayseri. This difference suggests that the prevalence may have increased over the years in our province. However, to reach a definite conclusion on the increase in prevalence, we believe that simultaneous research in the same provinces and comparison of the results will give more reliable results.

Many studies have been conducted on the epidemiology, prevalence, diagnosis and treatment options of hemoplasma infections. Willi et al. (2006) in a study on 713 cats concluded that cats that were not domesticated were more susceptible to hemoplasmas. Laberke et al. (2010) in their study to determine the risk factors for infection on 296 cats in Germany, reported that male, short-haired breeds and FeLV-infected cats with outside access were at higher risk of contracting the infection. Similarly, Sykes et al. (2008) and Tanahara et al. (2010) reported in their study that male cats and cats infected with FeLV and/or FIV, plus middle-aged cats with a history of fight wounds, were more likely to develop the disease. Roura et al. (2010) found no correlation between hemoplasma infection in cats and health status, age, breed, presence of anemia, FeLV and other vector-borne infections, but reported a high risk of infection in male and FIV-infected cats with outdoor access. In the Mhf positive-cats in our study, the prevalence rate for those that were kept domestically was 10.2 %, and that of those kept in the garden was 15.7 %. In consistence with some studies, there was no significant statistical difference between being kept domestically or outside and the rate of infection ($p>0.05$) (Table. 1) (Alexandre De Santis et al., 2014; Torkan et al., 2012).

Martinez-Diaz et al. (2013) reported that older and male cats had a higher prevalence in their study. However, in our study, as Atalay (2013) stated, the risk of infection related to gender could not be determined.

When the age-related incidence was examined, the incidence of Mhf was 10.0 % in those less than 1-year old and between 1 and 5-years old, and 40.0 % in those aged five and above. Thus, that of the aged group (5-years and above) was determined to be high (Table 3). Similarly, Do et al. (2020) also reported that the rate of hemoplasma in animals over 5-years old is higher (70 %) than in cats aged 1-5-years. Atalay (2013) made a different age grouping in a study and reported that the age factor is not important, but the risk of infection in cats above 4-years old is 2.082 times higher.

It has been reported that the risk of contracting Mhf is higher in patients with FIV, FeLV, rotavirus infection or immunosuppression (Sykes et al., 2007a; Georges et al. 2012). Jenkins et al. (2013) reported that 20 out of 62 cats they found positive for hemoplasma were infected with FIV and 11 positive for FeLV in their study on 200 cats, and these diseases increased the risk of developing hemoplasma infection. Nonetheless, Duarte et al. (2015) could not detect a relationship between FeLV and the risk of developing hemoplasma, but reported that the prevalence of hemoplasma is higher in cats with FIV infection.

Sykes et al. (2007a) found that cats infected with Mhf were less likely to be anemic than uninfected cats in a study conducted on 263 cats, and associated the disease with FIV seropositivity, cutaneous squamous cell carcinoma and stomatitis. In our study, the diagnoses of the cats that were positive for Mhf were in a wide range, and none of the diagnoses, including 3 cats with FIP, were evaluated in terms of the risk of the disease.

Clinical findings in hemoplasma infection differ according to the presence of another disease simultaneously, the type of causative agent or the stage of the infection (Harvey, 2006). Patients may be asymptomatic or carriers after clinical recovery (Kewish et al., 2004; Georges et al., 2012). Clinically, patients may experience symptoms that are not specific for hemoplasma infection such as weakness, depression, dehydration, mucosal pallor, lethargy, cardiac murmur, tachycardia, dyspnea, tachypnea, hepato-splenomegaly, lymphadenopathy, and intermittent fever (Braga et al., 2012). Maher et al., (2010) reported that the incidence of hemoplasma was higher in anemic cats, and some researchers stated that there was no significant statistical difference between the incidence of anemia and the disease (Sykes et al 2007 a; Pedrassani et al 2019). In our study, 3 of the PCR-positive cats and 6 of the PCR-negative cats were evaluated as anemic, based on the erythrocyte and hematocrit values and clinical findings.

The WBC, LYM, NEU, EOS, RBC, HCT, MCV and PLT values of the cats in our study were examined, and there was no statistically significant difference between

Mhf positive and negative cats. Pedrassani et al. (2019) found lymphopenia in one of 2 cats infected with Mhf in a study conducted on 30 cats. Although not statistically significant, lymphopenia was found in 2 cats and leukocytosis in 4 cats with breast tumor and FIP diagnosis in our study.

Since hemoglobin and MCH values could not be measured in three Mhf positive cats with low erythrocyte and hematocrit values, the type of anemia could not be determined. Despite that, it was diagnosed as anemia by considering the clinical findings together with the erythrocyte and hematocrit values. Atalay (2013) thought that the statistical difference between cats with and without feline infectious anemia in terms of HGB value and HCT value might be due to the chronicity of the infection.

In conclusion, our study is the first incidence study on cats in the Kırıkkale province. According to the PCR analysis findings, the incidence of Mhf was determined as 13 %. It was determined that there was no significant statistical difference between cats kept domestically or outside, gender and the rate of infection.

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**Conflict of Interest**
The authors declare that they have no competing interests.

**Authorship contributions**
Concept: E.P., S.Y.D., Design: E.P., S.Y.D., Data Collection or Processing: E.P., S.Y.D., Analysis or Interpretation: E.P., S.Y.D., Literature Search: E.P., S.Y.D., Writing: E.P., S.Y.D.

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