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Research Article

Intense exercise stress may trigger *Corynebacterium kutscheri* infection in Sprague-Dawley rats

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ABSTRACT

Corynebacterium kutscheri infection in rodents is usually related to stressful conditions. In this study, *C. kutscheri* infection has been reported that occurred as a complication in rats subjected to intense swimming stress during an experimental study. Weight loss, indifference to the environment, fluffy feathers, and hunched posture were observed in affected rats. The lungs contained numerous, randomly distributed, variably sized, slightly raised, cream-colored caseopurulent foci. There were occasional weak adhesions between the lung lobes and the adjacent costal pleura. Multifocal to coalescing necro-suppurative pneumonia with intralesional scattered large colonies of bacteria was observed histopathologically. Adjacent in the pulmonary parenchyma, interalveolar hypercellularity, type II pneumocyte hyperplasia, fibrinonecrotic vasculitis, and pleuritis were observed. Brown-Breen staining revealed gram-positive cocobacilli in the lesion areas. Furthermore, cardiac lesions in which the atria were more severely affected than the ventricles were identified. This lesion was characterized by thickening of the epicardium with intense infiltrates of macrophages admixed with scattered neutrophils. In severely affected rats, this lesion was also involved to the underlying myocardium. Bacterial culture yielded positive growth for *C. kutsheri* from the lesioned organ. Polymerase chain reaction was used to confirm the presence of genetic material for *C. kutscheri*. As a result, it was revealed that Sprague-Dawley rats were infected with *C. kutscheri* due to intense exercise stress. Periodic controls of *C. kutscheri* have been suggested in units where experimental animals are raised, both because of its negative effects on the results of the studies to be conducted and because of its zoonotic nature.

INTRODUCTION

The disease caused by *Corynebacterium kutscheri*, also known as pseudotuberculosis, was first described by Kutscheri in 1984 in mice (Lemaistre & Tompsett, 1952). Later research revealed that the disease also affects rats, hamsters, and guinea pigs (Otto et al., 2015). *C. kutscheri* is a diphtheroid gram-positive bacillus. It can be observed in different morphologies during growth for this reason also called "Chinese letter" (Won et al., 2007). Natural infection with *C. kutscheri* is generally subclinical (Amao et al., 1995). Early studies showed that this organism was commonly harbored latently in colonies of rodents. Bacteria are commonly found in the oral cavity, cecum, and oropharyngeal, cervical, and maxillary lymph nodes (Amano et al., 1991; Amao et al., 2002; Amao et al., 2008). Although good laboratory practices reduce the prevalence of the infection, it can occasionally be encountered with fetal epizootics (Barthold, 2012). Concurrent stressors are frequently associated with disease outbreaks. Transportation, overcrowding, malnutrition, immunosuppressive drugs, radiation, and experimental applications are among these factors (Amao et al., 2008). Latent infections can become active in the presence of these factors. The active infection that results most likely spreads hematogenously to various tissues and organs, causing necrosuppurative lesions (Giddens et al., 1968). The location of the lesions varies depending on the animal species. The lungs are the most severely affected organ in rats. In mice, the liver is the primary active site of infection (Barthold, 2012). Clinical findings differ depending on the affected organ, but they are not specific (Otto et al., 2015). It is necessary to remove any latently infected animals discovered in experimental animal facilities through periodic analysis to prevent the spread of infection. The definitive diagnosis of the disease is made according to the presence of characteristic histolopathologic lesions, bacteriological culture, and molecular analysis results (Fox et al., 1987; Jeong et al., 2013).So far, no infections with *C. kutscheri* have been reported in experimental production facilities in Turkey. Moreover, no clinical, pathological, or microbiological studies have been conducted to investigate the presence of *C. kutscheri* in experimental animals. This could be due to a lack of research on the subject rather than a lack of latent *C. kutscheri* infection in rodent colonies. The lack of scientific research on this subject may have an adverse effect on the outcomes of animal experiments (Saltzgaber-Muller & Stone, 1986). Aside from these, *C. Kutscheri*'s zoonotic nature poses significant potential risks to the occupational health and safety of researchers and assisting staff working in laboratory animal breeding units (Himsworth et al., 2014; Holmes & Korman, 2007). The clinical, gross, histopathological, bacteriological, and molecular findings of *C. kutscheri*-associated disease in a colony of Sprague-Dawley rats in a certified experimental animal breeding unit are presented in this study. In this context, we aimed to draw attention to the *C. kutscheri* disease among researchers who will be working with experimental animals and their personnel in the experimental animal breeding unit.

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MATERIAL and METHODS

Animals

The study's animal material included Sprague-Dawley rats that died or were euthanized as a result of a *C. kutscheri* infection that occurred as a complication during an experimental study. During the experiment, the rats were housed in standard temperature (22 ± 2°C), relative humidity (50%–70%), and lighting conditions (12/12-h light/dark). Ad libitum access to standard rat feed and tap water was provided. Thirty-two 10-week-old Sprague-Dawley rats were obtained from a certified commercial experimental animal breeding facility for the experiment. The rats were randomly divided into two groups of 16 rats each: forced swim groups and control non-swimming groups. The rats in the forced swim group had a weight equal to 5% of their body weight attached to their tails and were subjected to 1 h of swimming in 32°C pools 5 days a week (Claudio et al., 2013). The rats were dried after flotation before being placed in their cages. The rats in the control group received no treatment.

In the forced swim group, 1 rat (no. 1) died on the 10th day of the experiment and 2 rats (nos. 2–3) died on the 11th day of the experiment. The study was halted because the systemic necropsy findings of the dead rats indicated that the experiment's results would be harmed. All of the rats were examined, clinical symptoms were recorded, and they were euthanized humanely via cervical dislocation under general anesthesia induced with xylazine and ketamine.

Pathologic examination

Systemic necropsies were performed on dead and euthanized rats, and gross and subgross lesions were recorded. Identified macroscopic lesions were photographed (#c5050, Olympus), and tissue samples were fixed in 10% buffered formalin for 48 h and trimmed according to the proposed method of Ruehl-Fehlert et al. (2003) before loading into tissue follow-up cassettes. Tissue samples were dehydrated by passing them through a series of increasing alcohol concentrations, then cleared with xylene, and embedded in paraffin. With a microtome, serial sections of 4–5 µm thickness were cut from paraffin blocks and stained with hematoxylin–eosin (HE). Brown-Breen staining was used to highlight the agents in selected tissue sections. The tissue slides were examined with a light microscope (#BX51, Olympus), and photomicrographs were taken with a camera (#SC180, Olympus).

Bacteriological examination

Under aseptic conditions, samples from the lung, submandibular lymph node, spleen, liver, and preputial glands of all rats were inoculated on Columbia Agar plates with 5% sheep blood (#110025, Liofilchem®) for bacteriological examination. In both aerobic and microaerophilic environments, the media were incubated at 37°C for 24–48 h. Bacterial microscopic properties were determined by staining isolated bacterial colonies with the gram staining method. The biochemical properties of the cultured bacteria were characterized with VITEK 2® (bioMérieux).

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the isolated *C. kutscheri* strains' molecular identities. Genomic DNA was isolated with the help of a High Pure PCR Template Preparation Kit (#11796828001, Roche) in line with the manufacturer's recommendations. Primers specific to C. kutscheri reported by Jeong et al. (2013) as (F: 5'-CGTGATGGCCATCTTTGGTT-3', R: 3'-AATCGTATTAGCAAAGGTATGC-5') were used. DNA was amplified with an Xpert Fast Hotstart Mastermix (2×) with dye kit (#GE45.0001, GRiSP) in a Techne TC-412 thermal cycler (Keison Products) using the following cycling conditions: 95°C hold 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 15 s, and final elongation at 72°C for 3 min. The PCR products were loaded onto a 2% agarose gel and run at 100 V for 1 h before being visualized.

RESULTS

Clinical signs

The forced swimming rats showed rapid weight loss (12/16, 75%), indifference to the environment (12/16, 75%), fluffy hairs (12/16, 75%), and hunched posture (12/16, 75%) 1 week after the experiment began. In addition to these symptoms, periocular encrusted exudate (7/16, 43.75%), wheezing (7/16, 43.75%), and gasping (7/16, 43.75%) appeared later. On 10th (n=1) and 11th (n=2) days of the experiment, the rats were seen motionless on the floor with subnormal temperature and superficial respiration and subsequent death by passing into an agony state.

Gross findings

The control rats had no macroscopic lesions. The lungs of 12 of the rats in the forced swim group were heavier, edematous, and poorly collapsed. Suppurative foci of 1- to 6-mm size, cream-colored, and raised from the surface were found in the lungs of seven of these rats (Figure 1A). Some lesions were seen merged together, encompassing the entire affected lobe of the lung. The location and extent of lesions in the lobes of the lungs has been shown in Table 1.

There was no specific pattern of lesions in the lung lobes, and lesions were randomly distributed. Multiple caseonecrotic foci of variable sizes were seen on the cross-sectional surface of the lesion areas. In one case (no. 4), pus could be seen oozing from the lesion's cross-sectional surface, which was surrounded by a thick fibrous capsule (Figure 1B). Serous-to-fibrinopurulent foamy fluid was present in the tracheal lumen in five cases. The pleura had a ground-glass appearance in six cases (nos. 1–3, 5–7), and adhesions formed between the lung lobes and the pericardium and thorax in three cases (nos. 5–7). The pleural appearance was granular in the areas of adhesion. The apex of the heart was rounded in six rats, the myocardium was white mottled, particularly in the atria, and both ventricle lumens had coagulated blood. The pericardium had a ground-glass appearance in all six of these cases, and there were adhesions between the pericardial leaves. The liver was swollen and cyanotic in six rats, with blood oozing from the cut surface.

Table 1. Location and degree of lesions in the lobes of the lungs

No.	Lung				
	Left lobe	Right lobe			
		Cranial	Middle	Caudal	Accessory
1	+++	+++	+++	+++	+++
2	+++	-	-	-	-
3	+++	+++	+++	+++	+++
4	-	-	-	+++	-
5	-	+++	++	+++	-
6	+	-	-	+	+++
7	-	+++	++	+++	-

Note. According to the degree of involvement of lung lobes:–, no lesion; +, affected <25%; ++, affected 25%–50%; +++, affected >50%.

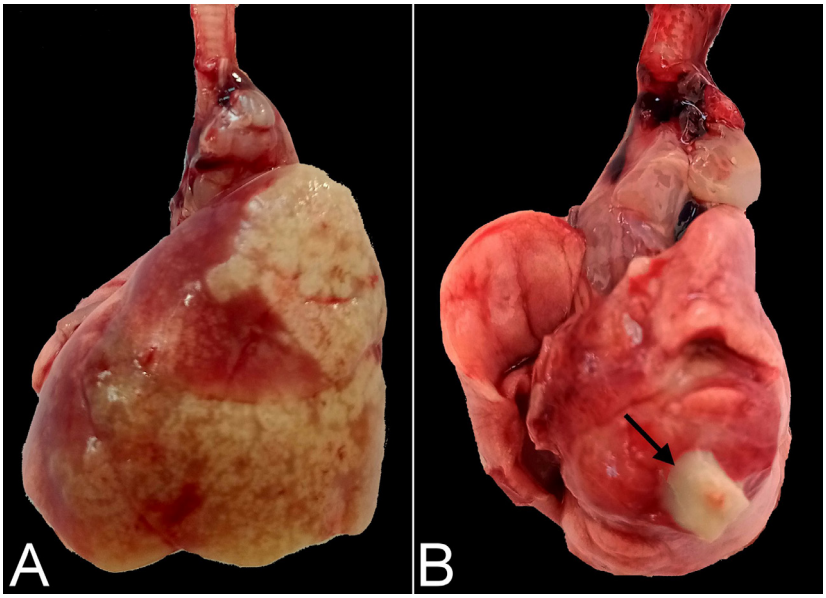

Figure 1. Corynebacterium kutsheri-associated gross lesions. A) Randomly distributed, varying sizes, slightly protruding from the surface, grayish-white caseo-purulent foci in the lungs; B) Single, large, raised abscess in the lung. Grayish-yellow pus oozing from the cross section of the lesion.

Histopathological findings

The lungs and hearts had the most visible microscopic lesions. In general, lung lesions were seen to begin with neutrophil infiltration to the interalveolar septum (12/16, 75%). At this stage, the alveolar lumens were empty (Figure2A).Hypercellularity in the interalveolar septum was occasionally accompanied by alveolar epithelization. As the lesions progressed, the alveolar lumens were seen to fill with inflammatory exudate containing shed epithelial cells, neutrophils, and macrophages (Figure 2B). In the lungs of seven rats, multifocal randomly distributed coagulative to caseous necrotic foci with abundant viable and degenerate neutrophils were observed (Figure 2C). Scattered colonies of basophilic bacteria were observed in the necrotic areas. Using Brown-Breen staining, these bacterial colonies in the necrotic areas were identified as gram-positive coccobacilli. Around neutrophil collections, macrophages, epithelioid histiocytes, and sporadic multinucleated giant cells were found on occasion (Figure 2D). Merging neighboring lesions resulted in larger lesions in severely affected lung lobes. In the immediate vicinity of the caseonecrotic foci, neutrophils with karyorrhectic nuclei formed a demarcation zone. (Figure 2E).

In one rat (no. 4), the lesions were surrounded by mature connective tissue from the outside. Bronchial, bronchiole, and tracheal epithelium was exfoliated in cases where the lung lobe was diffusely affected, and fibrinopurulent exudate covered the ulcerated mucosa. The vessels near the pyogranulomatous lesions had fibrinonecrotic vasculitis and occasional thrombosis (Figure 2F-G).

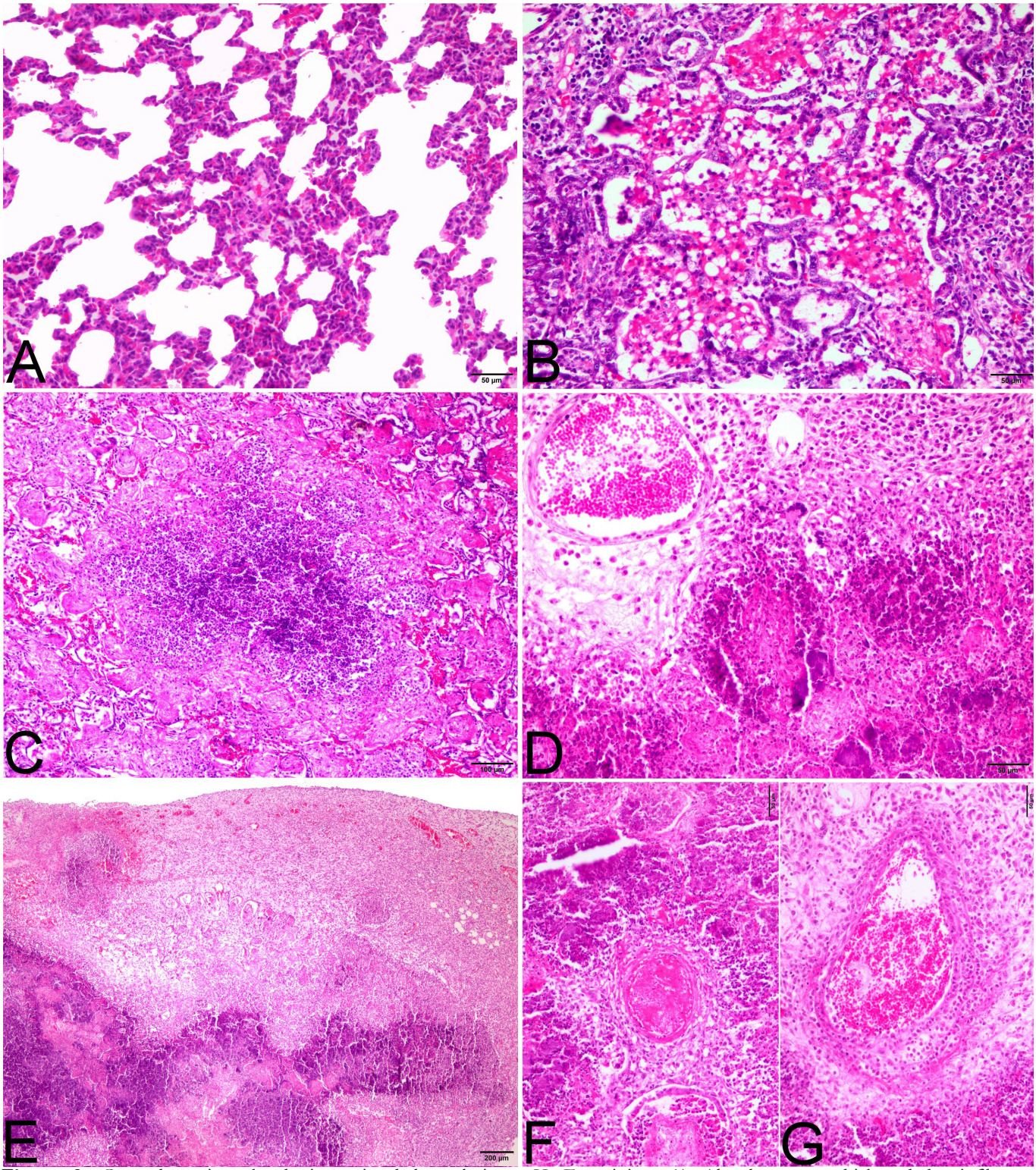

Figure 2. Corynebacterium kutsheri-associated lung lesions. H&E staining. A) Alveolar septa thickened by infiltrating neutrophils; B) Alveolar surfaces covered by cubic shaped pneumocytes (type II pneumocytes). Alveolar lumina contain homogenous edema fluid mixed with neutrophils; C) Note dark basophilic infiltrates of viable and degenerated neutrophils in a background of proteinaceous edema fluid; D) Accumulation of viable and degenerated neutrophils with intralesional large colonies of bacteria; E) Large coalesing areas of necrosis, infiltrated and surrounded by a variable amounts of neutrophils admixed with scattered macrophages. Pleura expanded by immature connective tissue composed of a mixture of proliferating fibroblast, new vessel formations and abundant macrophages, with occasional neutrophils; F) Hyalinization of the vessel wall and thrombosis in its lumen; G) Arteriole expanded with infiltrating neutrophils, macrophages and edema fluid.

The pleural surface was covered with a layer of fibrin with varying amounts of neutrophilic infiltrate in six cases (nos. 1–3 and 5–7). In addition to the fibrin tag, three rats (nos. 5–7) had increased pleural surface thickening with immature connective tissue composed of proliferating fibroblasts, neovascularization, abundant macrophages, and occasionally neutrophils. Six cases (nos. 1–3 and 5–7) had cardiac lesions that began in the epicardium and spread to the myocardium (Figure 3A-D). The atria were affected more severely than the ventricles. Furthermore, the severity of the lesions was greater on the right (5/6) than on the left side of the heart. The epicardium was markedly expanded by intense infiltrate of macrophages admixed with scattered neutrophils, lymphocytes, and mast cells. Cardiac myocytes are separated, surrounded, and lost in the subepicardial regions, with replacement by the inflammatory infiltrates described above.

Bacteriological findings

A pure culture of gram-positive coccobacilli was isolated from the affected organs. Based on biochemical properties, the organism was identified as *C. kutscheri*. In the PCR analysis, an isolate of 540 bp specific to *C. kutscheri* was obtained, which was consistent with this expectation (Figure 4). The non-spe-

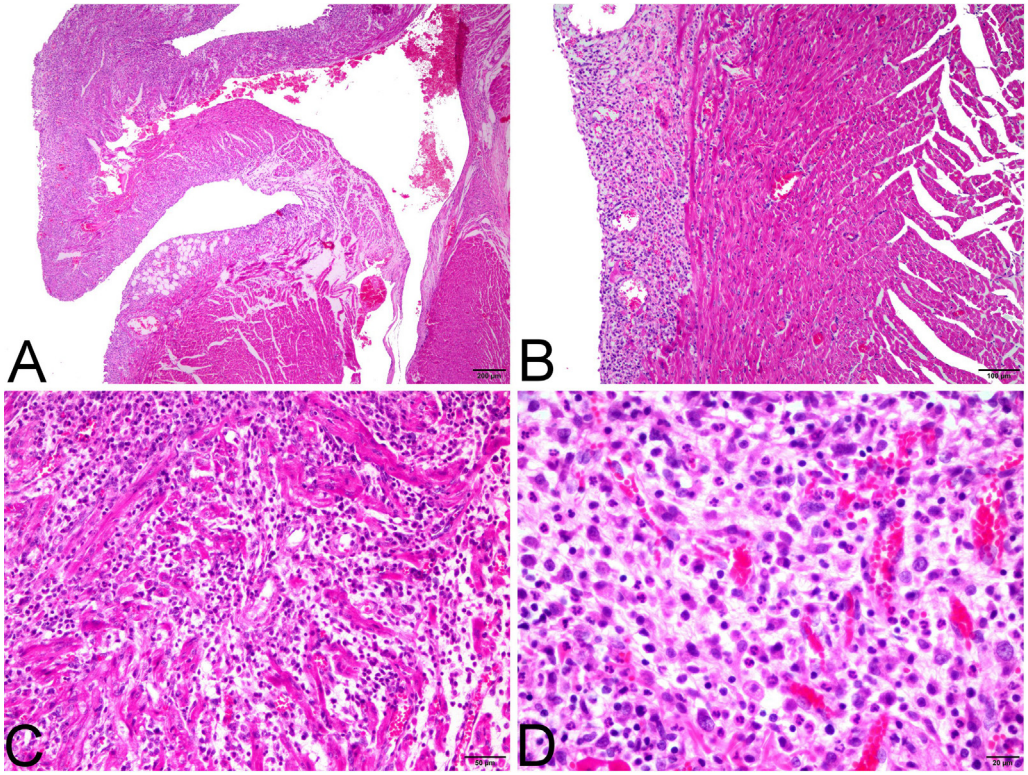

Figure 3. *Corynebacterium kutsheri*-associated cardiac lesions. H&E staining. A-B) Epicardium was markedly expanded by inflammatory infiltrates. These lesions spreading to the subepicardial myocardium. Note that the severity of atrial lesions is greater than that of the ventricle; C-D) Close-up images of the lesions. Cardiac myocytes are separated, surrounded, and lost with replacement by intense infiltrate of macrophages admixed with scattered neutrophils, lymphocytes and mast cells. Loss of cross striation in remaining cardiomyocytes.

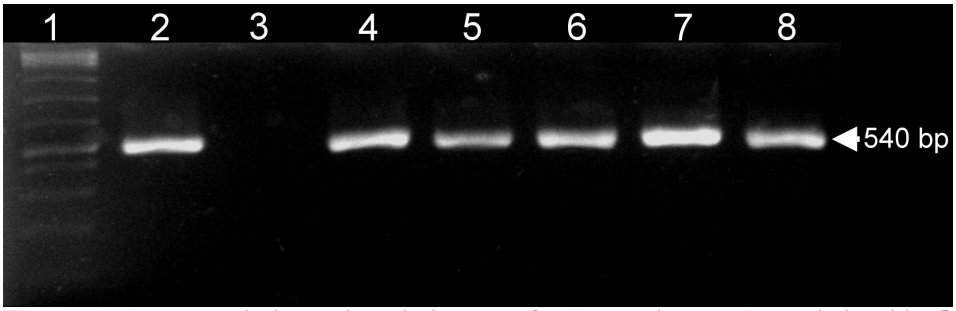

Figure 4. Agarose gel electrophoresis images of PCR products. PCR analysis with *C. kutsheri*-specific primers showing a 540 bp product as expected. Lane 1, 100-bp DNA ladder; Lane 1: positive control; Lane 2: negative control; Lane 3-7: tissue samples.

cific product was not determined. Based on the results of the bacteriological, biochemical, and PCR analyses,the causative agent of the disease was identified as *C. kutscheri*.

DISCUSSION

Corynebacteriosis, caused by *C. kutscheri*, was one of the first rodent diseases to be identified (Lemaistre & Tompsett, 1952). *C. kutscheri* has been found in the majority of rodent colonies studied in various countries (Otto et al., 2015). Despite reports that the measures taken have reduced the prevalence of infection, recent disease outbreaks indicate that *C. kutscheri* infection remains a threat to rodent colonies (Barthold, 2012; Won et al., 2007). In our literature review, we found no reports of *C. kutscheri* infection in rodent breeding units in our country. *C. kutscheri* infection is typically subclinical (Amao et al., 1995). As a result, it is critical to detect and exclude rodents infected with *C. kutscheri* prior to beginning experimental studies to obtain healthy results. Furthermore, outbreaks of clinical disease encountered during an experimental study, as in this study, may necessitate the study's termination, resulting in even greater losses (Saltzgaber-Muller & Stone, 1986). The clinical, macroscopic, histopathological, and bacteriological aspects of *C. kutscheri* infection, which emerged as a complication during an experimental study, were discussed in this study. This is the first study in our country to report *C. kutsheri* infection.

Infection with *C. kutscheri* in rodent colonies ranges from latent infections to fetal epizootic of pseudotuberculosis. Although tissue lesions are not asociated with latent infections, *C. kutscheri* can be isolated from many tissues of latently infected rodents (Barthold, 2012). Previous reports suggest that domestic animals become more susceptible to the diseases due to stress factors (Hazıroğlu et al., 2006; Tunca & Hazıroğlu, 2004; Tunca et al., 2006). *C. kutscheri associated* outbreaks are often caused by immune suppression under stressful conditions. Previous research has shown that immunosuppressive factors, such as malnutrition, transportation, overcrowding, high ammonia concentrations, and exogen administariton of immunosuppressive agents, such as cortisone and cyclophosphamide, can activate latent infections, leading to clinical disease (Shechmeister & Adler, 1953; Won et al., 2007; Zucker & Zucker, 1954). In this study, the rats that developed disease from *C. kutscheri* were not exposed to any of the stress factors listed above. It was interesting to see that the disease only appeared in rats exposed to swimming stress. There was no pathology found in the rats that were not swimming. Physical exercise, for example, activates adaptive responses, such as the sympathomedullary pathway and the hypothalamus–pituitary–adrenal axis, which release stress hormones to end the stress reaction and maintain homeostasis (Caplin et al., 2021). However, the excessive amount of stress hormones released during intense exercise may cause immunosuppression by suppressing bone marrow (Kruk et al., 2020). The emergence of disease only in the forced swim group in our study suggests that the stress induced by swimming contributed to the development of clinical disease by activating an existing latent infection under immunosuppression.

Clinical signs of *C. kutscheri* are often seen in the last phase of the disease (Barthold et al., 2012; Otto et al., 2015). Cachexia, hunched posture, periocular crusting, apathy to the environment, and respiratory distress reported in spontaneous or experimental *C. kutscheri* infection (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015; Won et al., 2007) were also seen in this study. Porphyria and lameness reported by Otto et al. (2015) were not seen.

The location of lesions in *C. kutscheri* differs depending on the animal species; consistent with previous reports (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015; Won et al., 2007), the majority of active infection was seen in rats' lungs. This study also found fibrinous pleuritis and mediastinal lymph node enlargement in addition to the pulmonary lesions seen in previous natural and experimental *C. kutscheri* infections (Giddens et al., 1968; Won et al., 2007). However, necrosuppurative hepatitis reported by Tuffery and Innes (1963) was not seen. It is unknown how the agent enters the lungs during active infection. The predominance of pulmonary lesions points to inhalation of contaminated aerosols as a possible mode of transmission. However, it is now widely accepted that the agent enters the lung via the hematogenous route (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015). In our cases, lung lesions began at the interalveolar septum rather than the bronchioloalveolar border. This finding indicated that the agent entered the lungs through the hematogenous route rather than through inhalation. Furthermore, the random distribution of the pulmonary lesions in all lobes rather than just the cranial lobe (lesion limited to cranial lobe indicates spread by inhalation) supports the hematogenous route of transmission. As the disease progresses, diffuse involvement of the affected lung sections and pleuritis indicate that it spreads via the pericanalicular route. The lungs are affected in mild cases, but the airways are unaffected; however, severe lung lesions are accompanied by bronchial, bronchiolar, and tracheal lesions. This finding suggested that intracanalicular spread occurred later in the process. As a result, the agent was thought to have entered the lungs via the hematogenous route and spread via the pericanalicular and intracanalicular routes.

The first histopathological findings in *C. kutscheri* infection are foci of interstitial necrosis with neutrophil infiltration (Barthold et al., 2012; Otto et al., 2015). In line with this, we discovered that pulmonary lesions in rats infected with *C. kutsheri* began in the interstitium. Granulomatous foci in the lungs of *C. kutscheri*-infected rats were thought to be the first histopathological lesion in rats resistant to this infection (Giddens et al., 1968). However, neither a later study (Won et al., 2007) nor our findings support this hypothesis. It has been reported that initially observed interstitial lesions accompanied by neutrophil leukocytes enlarged and became surrounded by neutrophils and macrophages to form abscesses. In chronic cases, abscess foci are surrounded from the outside by a fibrous capsule (Barthold et al., 2012; Otto et al., 2015). Similarly, in *C. kutscheri*-infected rats, the most visible lung lesion was multifocal randomly distributed foci of colliquation necrosis with numerous viable and degenerated neutrophils. Neighboring lesions tended to merge into larger lesions. A demarcation area formed by neutrophils with karyorrhectic nuclei separated these lesions from the surrounding tissue. These lesions were surrounded by epithelioid histiocytes and giant cells in chronic

infection and only rarely by a fibrous capsule. Lung lesions were classified as pyogranulomatous lesions in this regard.

The pathological findings of *C. kutsheri* infections is non-specific (Barthold et al., 2012; Otto et al., 2015). Therefore, bacterial isolation via culture or PCR demonstration of *C. kutscheri*-specific gene(s) is important in the diagnosis (Fox et al., 1987; Jeong et al., 2013). However, the fact that C. kutscheri can be isolated from many tissues of latently infected rodents makes microbiological analysis challenging for disease diagnosis (Amano et al., 1991; Brownstein et al., 1985; Yokoiyama et al., 1975). Culturing the agents from the lesioned organs allows for a definitive diagnosis of the disease (Fox et al., 1987). The disease was diagnosed in the present study by isolating the agent from the lesioned tissues using bacteriological culture and demonstrating the genetic material of *C. kutscheri* in these lesions using the PCR technique. Moreover, using the Brown-Breen staining technique, a histochemical staining method, gram-positive bacteria were identified in the centers of the lesioned organs, allowing for a definitive diagnosis.

C. kutscheri has been isolated in various tissues and organs of subclinically infected rodents, including the oral cavity and submandibular lymph nodes (Amano et al., 1991; Brownstein et al., 1985). *C. kutscheri* has also been isolated up to day 90 from mice experimentally inoculated via the oronasal route (Yokoiyama et al., 1975). *C. kutschseri* infection, which was activated during an experimental application in rats and caused the development of pathological disease, was defined in this study. *C. kutscheri*'s zoonotic significance has grown in recent years. *C. kutscheri* has been isolated in cases of rat-bite fever caused by rodent bite, according to reports (Himsworth et al., 2014; Holmes & Korman, 2007). The present study revealed the disease threat faced by laboratory workers and animal caretakers in an experimental study due to the activation of *C. kutscheri* infection in rats subjected to swimming stress. Given the subclinical nature of *C. kutscheri* infections in rodent colonies, the study suggests that laboratory personnel should always take the necessary precautions when working with laboratory animals.

It has been reported that rodent species differ in their susceptibility to *C. kutsheri* infections. Intravenous administration of *C. kutscheri* to 15 different mouse breeds resulted in active infection in only seven of the breeds, with the remaining breeds remaining disease-free (Pierce et al., 1964). Although the exact cause of this difference between mouse breeds is unknown, Hirst and Campbell (1977) proposed that it is due to the mononuclear phagocytic system. They concluded that the monosystem phagocytic system effectively cleared the agent in resistant strains. Although there are studies presented in terms of susceptibility to the disease among mouse breeds, there is no study on which breeds are more susceptible in rat breeds. The Wistar breed rats were resistant by LeMaistre and Tompsett (1952). Infection occurs in these breeds only at high doses. Natural and experimental diseases have been observed in Sprague-Dawley rats in subsequent studies (Giddens et al., 1968; Won et al., 2007). The unavailability of reports of *C. kutscheri* infection in other breeds may be due to experimental studies' preference for Sprague-Dawley and Wistar breeds rather than disease resistance in these breeds. Natural *C. kutscheri* infection in Sprague rats was determined in the study under stress conditions caused by excessive exercise. However, active infection with *C. kutscheri* did not occur in all of the rats exposed to exercise stress. As a result, some of the rats exposed to stress developed the disease, whereas others did not, implying that, in addition to immunosuppression, individual differences play a role in disease development.

CONCLUSION

A natural *C. kutscheri* infection in Sprague-Dawley rats under stressful conditions induced by intense exercise was described in detail in terms of clinical, pathological, and microbiological perspectives in the present study. The subclinical course of *C. kutscheri* infections highlights the importance of routine *C. kutscheri* screening in experimental animal breeding facilities. *C. kutscheri*'s zoonotic significance suggests that staff working with experimental animals should pay special attention to this subject.

DECLARATIONS

Ethics Approval

All the experimental procedures were approved by the local ethics committee of Aydın Adnan Menderes University (approval no. 64583101/2021/091).

Conflict of Interest

The authors declare that no commercial funding was obtained that may be construed as potential conflict of interest.

Author contribution

Idea, concept and design: Eİ, ETE, RT

Data collection and analysis: Eİ, ETE, ÇN, RT

Drafting of the manuscript: Eİ, ETE, RT

Critical review: Eİ, RT

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Data Availability

The data are available from the corresponding author on reasonable request.

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NA.

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