Panophthalmitis in a Gentoo Penguin (*Pygoscelis Papua*) From The Antarctic Peninsula: Evaluation of Microbiological and Histopathological Analysis Outcomes

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Abstract

In this study, it was aimed to present the results of microbiological, cytological, histopathological, and immunohistochemical analyses of ocular samples from an Antarctic (Ardley Island, King George Island) Gentoo penguin chick (*Pygoscelis papua*) with pyogranulomatous panophthalmitis in the right eye. Samples were taken from both the healthy left eye and the lesion in the right eye. Conventional culture methods and phenotypic and molecular tests were used for bacterial isolation and identification, respectively. None of the isolates could be identified phenotypically. As a result, four of the five isolates obtained from the right eye were considered to belong to putative novel bacterial species and taxa as their similarity to GenBank data was below 98.75%. The isolates were considered to be *Pasteurellaceae* bacterium, *Corynebacterium ciconiae*, *Cardiobacteriaceae* bacterium, *Actinomyces* sp., and *Dermabacteraceae* bacterium. The only isolate from the left eye was identified as *Psychrobacter pygoscelis*. The cytological analysis demonstrated cell infiltrates composed mostly of degenerate heterophils, reactive macrophages, plasma cells, lymphocytes, and eosinophils. Based on histopathological findings, the lesion was defined as typical pyogranulomatous panophthalmitis. Immunohistochemistry demonstrated that the granuloma was positive for TNF- α , IL-4, MMP-9, IL-1 β , and IL-6. This is the first documented report of unilateral pyogranulomatous panophthalmitis in a Gentoo penguin chick, living in its natural habitat in Antarctica. This report also describes the isolation of four bacteria from the infected eye, which are considered to belong to novel Genus, species, or taxa. The primary bacterial pathogen that caused the ocular lesion was not able to be detected and remains unclear.

Introduction

Gentoo penguins (*Pygoscelis papua*) have a circumpolar distribution, and usually nest in small colonies on the sub-Antarctic islands and Antarctic Peninsula (González-Acuña et al. 2013). Since 2000, this species has been listed as near-threatened and only very limited information is available on its bacterial diseases (Barbosa and Palacios 2009; González-Acuña et al. 2013).

Eyesight is of critical importance for penguins as it aids in migration, orientation, and foraging (Nesterova et al. 2010). Thus, vision disorders adversely affect the capability of these animals to adapt to their physical and social environment. While numerous bacteria, viruses, fungi, and parasites have been isolated from diseases of the avian ocular surface (Griggs 2019), only a few studies are available on the ophthalmic parameters of several penguin species, including ocular bacterial flora, intraocular pressure, and tear production (Suburo et al. 1988; Martin 1999; Swinger et al. 2009; Nesterova et al. 2010; Bliss et al. 2015; Sila et al. 2015). In a study conducted by Swinger et al. (2009), the ocular bacterial flora and ophthalmic parameters of 28 captive penguins kept at a zoo were investigated. However, to our knowledge, there is no published study on the ocular infections of penguins.

In general, cultural methods and histopathological, cytological and immunohistochemical analyses are employed to diagnose and determine the aetiology of ocular infections, and most often these different methods are used together.

Isolation of bacteria from cases of infection is carried out by cultural methods. The isolates recovered are identified at the species level by means of phenotypic tests and molecular analyses (Quinn 2011). It is

common to use 16S rRNA gene sequencing for molecular analysis. Especially when identifying novel bacteria species, the cut-off value is set as 98.70% (Chun et al. 2018; CLSI 2018). Panophthalmitis is an inflammation of all layers of the eye, including the intraocular structures (Chen et al. 2017). Reports of ocular cytology findings are rare in the field of veterinary medicine, except for reports related to canine, feline, and equine conjunctival cells (Barnes and Grahn 2007; Perazzi et al. 2017; Bayram et al. 2019; Proietto et al. 2019), which are well-defined. As a quick-resulting technique, which produces good quality preparations, exfoliative cytology can be used efficiently together with bacterial culture for the diagnosis of ocular infections. Ocular infections are associated with increased levels of proinflammatory cytokines, such as IL-1 β , IL-1 α , IL-6, IL-8, TNF- α , and certain proteolytic enzymes (matrix metalloproteinases, MMPs) in the lacrimal fluid.

This study was aimed at presenting the results of the microbiological, cytological, histopathological, and immunohistochemical analyses of ocular samples taken from a Gentoo penguin chick (*Pygoscelis papua*) with pyogranulomatous panophthalmitis. These samples were taken from the right and left eyes of a penguin by Professor Latife ÇAKIR BAYRAM during the Third National Antarctic Scientific Expedition (TAE3), made to Antarctica (Ardley Island, King George Island) within the framework of the bilateral cooperation project signed between Turkey and Chile.

Materials And Methods

Ocular Samples

The ocular samples used in this study were taken from a Gentoo penguin (*Pygoscelis papua*) between 4 January and 9 February 2019, on King George Island, Ardley Island in Antarctica (62° 13' S; 58° 54' W) (Fig. 1). A bilateral cooperation project titled “*The cytological, microbiological and ophthalmic evaluation of samples of ocular surface from the Antarctic Peninsula penguins*” (Instituto Antártico Chileno, INACH, Antarctic Scientific Expedition, ECA 55) was agreed to be implemented under the framework of the Third National Antarctic Scientific Expedition (TAE3), made in the 2018-2019 period and organised by the Polar Research Centre (PolRec) of Istanbul Technical University. The ocular samples examined in the present study were collected at the location indicated above.

Approvals regarding entrance to the specially protected area of the Antarctic (Antarctic Specially Protected Area, ASPA, No: 150, Permit N°- 21- 2019) and for collecting eye samples from the penguins (Antarctic Specially Protected Area, ASPA, No: 150, Permit N°- 07- 2019) were provided by the Turkish Antarctic Program.

Capture and handling of the penguin

The capture and handling of the penguin were performed as described by González-Acuña et al. 2013 and in accordance with the standard methods laid down for the Ecosystem Monitoring Programme by the Commission for the Conservation of the Antarctic Marine Life Resources (CCAMLR 2004). For the sampling procedure, the penguin was restrained in an upright vertical position by applying gentle pressure to its wings, base of skull, and beak. During sampling, the penguin was maintained manually in a facedown position.

Sampling

In situ examination of the right eye demonstrated the presence of a reddish-orange coloured, granulation tissue-like, proliferative and exuberant lesion on the ocular surface. The movement of the nictitating membrane was not observed due to this lesion. The proliferative tissue was determined to occupy the cornea and other parts of the eyeball (Fig. 2). An extensive exuberant mass, which occupied the anterior chamber, made it impossible to identify the intraocular structure. No macroscopic lesion was observed in the left eye.

Using a sterile scalpel, samples were taken from the mass in the right eye into 1 ml of sterile distilled water for microbiological analysis, Cytospin collection fluid (Cytobrush, Gynobrush Plus, Germany) for cytological analysis, and 70% ethyl alcohol for histopathological analysis. The left eye of the penguin, which appeared to be healthy, was sampled for microbiological and cytological analyses. The microbiological samples were collected with a swab into Stuart's transport medium, whilst the cytological samples were collected either by brushing into Cytospin collection fluid or by the membrane filter technique (cellulose-acetate filter papers; Sartorius, HAWG 047 AO, 3x4 mm in diameter, 0.45-micrometer pore size) into 70% ethyl alcohol (Bolzan et al. 2005; Singh et al. 2005). The swab and brush were gently rolled over the cornea on the limbus, such that the palpebral and forniceal conjunctiva, anterior to the third eyelid, were also sampled.

Filter strips with a pointed tip on one end were placed on the superior, ventral temporal bulbar conjunctiva, near the limbus as described by Bolzan et al. (2005) and Singh et al. (2005) with minor modifications. The strips were removed with a peeling motion, and the samples were immediately transferred to the laboratory at the Professor Julio Escudero Base, King George Island, Antarctica. The samples were stored at 4°C and examined within 2 weeks.

Bacteriological analysis

The ocular tissue sample taken into distilled water for bacteriological analysis was broken into small pieces by shaking. Slides were prepared from the resulting suspension, and were stained with the Gram and Ziehl-Neelsen techniques. 50 µl of the suspension was directly inoculated onto three plates of blood agar (Blood agar base No: 2, CM0271, Oxoid, Waltham, MA, USA/containing 7% sheep blood) added 2% of NaCl (Merck, Germany). The swab sample taken from the left eye was directly inoculated onto three blood agar plates. The inoculated plates were incubated at 37°C under aerobic, microaerobic and anaerobic conditions for 2-3 days. At the end of the incubation period, the colonies were examined both macroscopically (for size, colour, odour, haemolysis, etc.) and microscopically. Each different type of colony that had grown on the blood agar was subcultured onto sheep blood agar for purification. Both phenotypic tests (Gram staining, catalase, oxidase, motility and carbohydrate fermentation tests), as described by Quinn et al. (2011), and molecular analyses (16S rRNA gene sequence analysis) were performed for the identification of the recovered isolates at the species or genus level. The recovered isolates are stored in Brucella broth containing 15% glycerine at -84°C, as part of the culture collection of the Microbiology Department of Erciyes University, Faculty of Veterinary Medicine.

Mycological analysis

For the detection of possible fungal agents, samples from both eyes were inoculated onto Sabouraud dextrose agar (SDA, CM0041, Oxoid, United Kingdom). The inoculated plates were incubated at 22°C and 37°C under aerobic conditions for 2-3 days.

Molecular identification

DNA extraction

The DNeasy UltraClean Microbial Kit (12224-50, Qiagen, USA) was used for DNA extraction from the bacterial isolates.

16S rRNA sequencing

The species identification of the isolates was made by means of 16S rRNA gene sequence analysis. Accordingly, four primers, including 27F and 1492R, and two internal primers 518R: ATT ACC GCG GCT GCT GG and 968F: AAC GCG GAA GAA CCT TAC) were used for the sequencing of the 1465 bp-fragment of 16S rRNA. The PCR mixture used for the amplification of this 1465 bp-fragment was 25 µl in volume and contained 12.2 µl of molecular grade deionised water, 2.5 µl 10x PCR buffer, 2.5 µl MgCl₂ (25 mM), 0.5 µl 10mM dNTP, 1 µl of the forward primer (10 pmol), 1 µl of the reverse primer (10 pmol), 0.3 µl Taq DNA polymerase, and 5 µl of the DNA recovered from the isolates (25 ng/µl). Amplification was performed as follows: initial denaturation at 94°C for 4 min followed by 35 cycles including denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec, primer extension at 72°C for 1 min, and a final extension at 72°C for 6 min. The PCR products were run on agarose gel electrophoresis and those, which were of the appropriate size (1465 bp) and underwent 16S rRNA sequence analysis. The 16S rRNA gene was manually aligned with type strain sequences retrieved from EzBioCloud database (Yoon et al. 2017) following BLAST searches for fast identification.

Evolutionary relationships of taxa

Pairwise sequence similarities were calculated using the method recommended by Meier-Kolthoff et al. (2013b) for the 16S rRNA gene available via the GGDC web server (Meier-Kolthoff et al. 2013a) available at <http://ggdc.dsmz.de/>.

Phylogenies were inferred by the GGDC web server (Meier-Kolthoff et al. 2013a) available at <http://ggdc.dsmz.de/> using the DSMZ phylogenomics pipeline (Meier-Kolthoff et al. 2014) adapted to single genes. A multiple sequence alignment was created with MUSCLE (Edgar, 2004). Maximum likelihood (ML) and maximum parsimony (MP) trees were inferred from the alignment with RAxML (Stamatakis 2014) and TNT (Goloboff et al. 2008), respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion (Pattengale et al. 2010) and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were checked for a compositional bias using the X² test as implemented in PAUP* (Swofford 2002).

Antibacterial susceptibility testing

The susceptibilities of the bacterial isolates to amoxicillin/clavulanic acid (AMC, 30 µg), ampicillin (AM, 10 µg), azithromycin (AZM, 15 µg), enrofloxacin (ENR, 5 µg), gentamicin (CN, 10 µg), levofloxacin (LEV, 5 µg), and tetracycline (TE, 30 µg) were determined using the disc diffusion method (Clinical and Laboratory Standards

Institute, 2018). The antimicrobial discs were purchased from Bioanalyse, TURKEY. Due to we could not identify the isolates at the species level, the disk diffusion test results of our isolates were evaluated by using the criteria published by the Clinical and Laboratory Standards Institute (CLSI 2018 and CLSI 2019) for *Enterobacteriaceae* and staphylococci, respectively. *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as reference strains for the susceptibility tests.

Exfoliative Cytology

Cytospin Technique

The tissue samples taken from the proliferative mass into Cytospin collection fluid for cytological analysis were agitated on a vortex mixer. Cytoцентрифугed slides were prepared from this agitated suspension. The brush sample taken from the left eye was also transferred into a fixative solution (Cytospin collection fluid, Thermo Shandon 6768001, USA).

With an aim to evaluate cell morphology, Cytospin slides were prepared from ocular surface samples by cytoцентрифугation (Cytospin 4, ThermoShandon, Pittsburgh, PA, USA) at 1000 rpm for 5 min, using disposable plastic chambers (Cytofunnel, ThermoShandon) and glass slides (Cytoslide, ThermoShandon). Wet cytoцентрифугed slides were dried and then stained with the Haematoxylin-Eosin (HE) (Scott and Penny 1993) and Diff-Quik methods (Richard Allan Scientific, Thermo Electron Corporation, 9990700, USA). The slides were mounted with mounting medium (Merck, Germany).

Impression Cytology

This method was performed as described by Bolzan et al. (2005) with minor modifications. Briefly, the strips were immediately placed in individual Eppendorf tubes containing 95% ethyl alcohol. Next, the strips were stained with Periodic Acid-Schiff (PAS) x Haematoxylin, as described by Nelson et al. (1983) with some modifications such as the use of a filter with a pore size of 0.45 µm and sodium metabisulfite. The samples were hydrated with distilled water for 5 min, immersed in 0.5% periodic acid for 5 min, washed in distilled water for 5 min, immersed in Schiff's reagent for 5 min, washed under running water for 5 min, immersed in sodium metabisulfite (three successive immersions, each for 5 min), washed under running water for 10 min, counterstained with Harris' haematoxylin for 1 min, and washed under running water for 5 min. After these steps, the samples were dehydrated in 95% and 100% ethyl alcohol (two successive immersions, each for 1 min) and immersed in xylene (two successive immersions, each for 5 min). Finally, they were mounted onto slides in a way that the cells were faced upwards, and were cover-slipped using mounting medium (Bolzan et al. 2005).

Histopathological analyses

Tissue samples underwent routine processing and were embedded in paraffin. Nine 5- to 6-µm-thick serial tissue sections, representative of the entire tissue sample, were cut from the paraffin block and mounted onto glass slides. Each paraffin section was stained with H&E, and the Brown-Brenn Gram (Pritt et al. 2006), Grocott methamine silver stain (Polyscience) (Wright et al. 2017), acid-fast stain, and immunohistochemical stains. Furthermore, the streptavidin-biotin peroxidase (SABP) complex method (Invitrogen, USA) was

performed using five different antibodies, including those against TNF- α , IL-4, MMP-9, IL-1 β , and IL-6. While Chicken Bursa Fabricius tissue sample was used as a positive control, Phosphate buffer solution (PBS) was used instead of primary antibody in the negative control slides. In addition, since there is no commercially produced antibody specific to penguins, antibodies that are routinely used for immunohistochemical staining in poultry were used in the current study. The primary antibodies used in the present study are shown in Table S1. Immunoreactions were visualized using aminoethyl carbazole chromogen (AEC, Invitrogen, USA) (Adhikari et al. 2018; Balouria et al. 2019). The immunostaining results were evaluated by scoring the intensity of colour development, and were graded as strong, moderate or mild staining, paying special attention to the pattern and distribution of positive immunostaining in the histological sections (Fedchenko and Reifenrath 2014).

All cytological, histopathological and immunohistochemical (IHC) slides were imaged with a digital camera (Olympus DP71) and digital programmers (DP Controller and the DP Manager) fitted to a microscope (BX-51, Olympus, Tokyo, Japan) (minimum of 4 fields per slide, using x10, x40, and x100 objective lenses).

Electron microscopic examination of the isolates

All isolates were imaged by the transmission electron microscopy (TEM) and scanning electron microscopy (SEM) for the examination of the individual cell morphologies, at Erciyes University Technology Research and Application Center (ERU-TAUM, Kayseri, Turkiye). For this purpose, 24-48 hour fresh cultures belong to isolates grown in the solid medium were used.

Results

Bacteriological findings

While the samples were found to be negative for acid-resistant bacteria by Ziehl-Neelsen staining, they were observed to contain numerous Gram-negative coccobacilli and Gram-positive pleomorphic bacilli by Gram staining. The culture method yielded a total of 6 isolates, 5 from the right eye, and one from the left eye of the penguin. The isolates recovered from the right eye were numbered as TAE3-ERU1, TAE3-ERU2, TAE3-ERU3, TAE3-ERU4, TAE3-ERU5, and the isolate from the left eye was numbered as TAE3-ERU6.

The phenotypic and physiological characteristics of the isolates are presented in Table 1.

16S rRNA gene sequence analysis

Based on the BLAST (basic local alignment search tool) and EzBioCloud database searches of the 16S rRNA gene sequencing results of the isolates recovered from the right and left eye demonstrated that 2 of 5 isolates could be done species-level identification. Other isolates, which were found below threshold (98.7%) with similarities rate of the type strain, could not be identified as species-level. 16S rRNA gene sequence similarity of our isolates is given in Table 2. Each sequence was deposited in the GenBank database with MT444110-MT444114, and MT674807 for TAE3-ERU1 - TAE3-ERU5 and TAE3-ERU6, respectively.

Mycological analysis

All of the samples examined were found to be negative for fungi.

Phylogenetic analysis

According to the phylogenetic analysis result;

- TAE3-ERU1 was determined to be close to Bisgard Taxon 14 and also this isolate could be a new genus in *Pasteurellaceae*
- TAE3-ERU2 was determined to be close to *Corynebacterium ciconiae* CCUG 47525 type strain.
- TAE3-ERU3 was determined to be close to *Rappaport* and *Suttonella* in the *Cardiobacteriaceae* family,
- TAE3-ERU4, determined to be close the *Actinomyces*,
- TAE3-ERU5, found to close in the *Devriesea* genus in the *Dermabacteraceae* family
- TAE3-ERU6 was determined to be close to *Psychrobacter pygoscelis* I-STPP5b type strain.

Detailed information about phylogenetic analysis was given below phylogenetic trees of isolates in Fig. S1- Fig. S6.

Antibacterial susceptibility testing

Antibacterial susceptibility tests showed that five of the isolates were susceptible to all of the antibiotics tested (AMC, AMP, TE, LEV, ENR, AZM and CN). On the other hand, isolate TAE3-ERU5, belonging to the family *Dermabacteraceae*, was determined to be susceptible to AMC, AMP, TE, LEV, ENR and AZM, and resistant to gentamicin.

Pathological findings

Cytological findings

Abundant heterophils, a few aggregates of degenerate heterophils, and numerous intra/extracellular Gram-positive filamentous and Gram-negative coccobacilli and bacilli were observed in the cytopsin slides, which were prepared from the proliferative mass in the right eye and stained with Periodic acid-Schiff (PAS), Haematoxylin and Eosin (H&E) and Papanicolaou (PAP) staining techniques (Figure S7 (A-I)). Mixed cell infiltration, composed of mononuclear cells and squamous epithelial cells, degenerate heterophils and macrophages, intracellular bacteria in the cytoplasm of macrophages and karyolytic heterophils, was detected. Bizarre cells with two large kidney-shaped nuclei were also observed.

Furthermore, distinct squamous conjunctival metaplasia, characterized by mucus, keratin, and pyknotic cells with an enlarged cytoplasm and a decreased nucleus/cytoplasm ratio, was observed in some areas. In the impression slides, only abundant heterophil leukocytes and bacteria were observed, and goblet cells were not detected

In the cytopsin slides prepared from the sample taken from the left eye, columnar epithelial cells, small and round conjunctival cells, and non-cornified squamous corneal epithelial cells, were common. It was observed that the nucleus/cytoplasm ratio of these eosinophilic cells had not altered and that the cells were similar to

each other in shape and size. The examination of the impression slides revealed numerous heterophils, lymphocytes, macrophages, goblet cells and extracellular bacteria. (Fig. S7 (A-I)).

Histopathological findings

Histopathological examination showed that the proliferative mass, characterized by necrotic debris, cell infiltration, and numerous pleomorphic and filamentous branching bacilli, and sulfur granules, had replaced all layers of the anterior chamber of the eye. It was observed that the surface epithelium of the cornea had completely disappeared and the stroma was necrotic. Dense inflammatory cell infiltration had extended to the deep stromal layer of the cornea. The anterior chamber was completely displaced by necrotic and pyogranulomatous lesion. Furthermore, fibroblasts and fibrocytes were found at significantly higher numbers in the anterior chamber of the eye. Histopathologically, the reddish-orange coloured proliferative mass was a typical pyogranuloma. The granuloma was mature and displayed extensive coagulation necrosis at the centre with multinucleated giant cells palisading along the necrotic line. These giant cells were surrounded by an infiltration of numerous macrophages, heterophils, lymphocytes and fibroblasts, and few plasma cells and eosinophils (Fig. 3 A-E). Brown-Brenn staining demonstrated the clustering of club-like organisms comprised of numerous pleomorphic and filamentous Gram-positive bacteria within sulphur granules, which were surrounded by neutrophils and lymphocytes. Also, few small Gram-negative coccobacilli were also observed. Grocott-Gomori methenamine-silver staining revealed the presence of branched agents, characteristic of actinomycosis (Fig. S8). Fungi, and based on the results of Ziehl-Neelsen staining, acid-fast bacteria were detected neither within nor in the periphery of the granuloma. Furthermore, dysplastic alterations, including large hyperchromatic nuclei and binucleated cells with an increased nucleus/cytoplasm ratio and an irregular nucleus, were also observed. A histopathological section representative of the proliferative mass is shown in Fig. 3 (F-Q).

Immunohistochemical findings

Our immunohistochemical results demonstrated positive staining for matrix metalloproteinase-9 (MMP-9), proinflammatory cytokines such as tumour necrosis factor-alpha (TNF α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6), and anti-inflammatory cytokine IL-4 (interleukin-4) (Fig. 3 (R-AB)). Immunoreactivity for IL-1 β , (Fig. 3R-S) and IL-6 (Fig. 3V, W) was observed to be much more intense.

In the conjunctival epithelial and stromal cells of the cornea, strong IL-4 immunoexpression was detected (Fig. 3T). Cytoplasmic immunoreactivity for IL-4 and MMP-9 was observed to be very strong in the regions of the conjunctiva where macrophages and giant cells and necrosis were intense. (Fig. 3Y, Z, X).

Moderate cytoplasmic immunoexpression of MMP-9 was detected in the epithelial cells of the cornea. While the granuloma, which was composed of heterophils, macrophages, and multinucleated giant cells, showed positive immunoreactivity for IL-1 β , TNF α , and IL-6, the immunoreactivity for these proinflammatory cytokines was moderate in the epithelial cells of the cornea and sclera (Fig. 3R, S; U-Y, AA, AB). Immunostained slides prepared from the brush sample of the left eye were used as a control for the evaluation of MMP-9, TNF- α , IL-4, IL-1B, and IL-6 positivity. The immunochemical staining results of the slides prepared from the right and left eyes are shown in Fig. 3R-AB.

Morphological findings of the isolates

The morphological appearance of the bacterial isolates by light microscope, Transmission electron microscope, and Scanning electron microscopy SEM are presented in Fig. 4.

Discussion

In the present study, an ocular lesion occupying the right eye of a Gentoo penguin chick, living in its natural habitat (Antarctic Peninsula), was defined as pyogranulomatous panophthalmitis, based on cytological, histopathological and immunohistochemical analyses. Bacteria, which were unable to be identified at species level, but classified under 5 different genera, and considered belonging to putative novel taxa, genera and species, were isolated from the ocular lesion. Furthermore, *Psychrobacter pygoscelis* was isolated from the seemingly healthy left eye of the same animal. To our knowledge, in respect of its particularities described, the present study is the first and only of its kind. The ocular lesions of non-captive raptors are generally caused by trauma (Labelle et al. 2012; Moore et al. 2018). Previous ophthalmologic research is known to have been carried out mostly in captive raptors, psittacines and penguins kept at zoos. Previous ophthalmologic studies, conducted in penguins, either professionally cared for at zoological institutes or kept at aquariums, have focussed on ophthalmological parameters such as tear production and intraocular pressure (Swinger et al. 2009; Bliss et al. 2015; Gonzalez-Alonso-Alegre et al. 2015; Sheldon et al. 2017). Ophthalmic lesions reported to have been detected in captive penguins are limited to conjunctivitis (Leigue et al. 2016), keratoconjunctivitis (Jencek et al. 2012), and cataract (Swinger et al. 2009; Bliss et al. 2015; Church et al. 2018). On the other hand, no information is available on ophthalmic lesions or infections in non-captive penguins living in their natural habitat, including the Gentoo penguin. Several diagnostic tests are required to be performed to determine the aetiology of ocular lesions and manage ocular diseases in avian species (Michalle Willis and Wilkie, 1999a, 1999b; Meekins et al. 2015; Griggs, 2019). Exfoliative cytology (cytospin centrifugation together with brush cytology and impression cytology) of the corneal and conjunctival epithelium is a routine complementary diagnostic method (Perazzi et al. 2017), which is successfully used in various animal species (Bolzan et al. 2005; Perazzi et al. 2017; Proietto et al. 2019). An initial diagnosis can be made by the detection of the cell population and the presence of infectious agents via exfoliative cytology (Bolzan et al. 2005; Cakir et al. 2014). In the present study, cytological analysis of the samples taken by the cytobrush and membrane filter techniques from the right eye of the penguin demonstrated ocular surface cells, infiltrating reactive cells and the presence of bacteria with different morphological features (Fig. S7). In agreement with the findings of previous reports for cytospin slides prepared from samples of animals with keratoconjunctivitis (Cakir et al. 2014), the present study revealed the presence of binucleated epithelial cells. The presence of binucleated cells is considered as an indicator of reactive changes in areas of severe chronic inflammation. On the other hand, the cytological analysis of the sample taken from the left eye of the same animal demonstrated normal ocular surface cells, similar to descriptions made in previous literature reports (Bolzan et al. 2005; Athanasiou et al. 2018). In the present study, based on histopathological examination (Fig. 3), the lesion in the right eye of the penguin was defined as pyogranulomatous panophthalmitis. To the authors' knowledge, there is no previous report on the diagnosis of panophthalmitis in captive or non-captive penguins. However, reports have been published on *Pseudomonas aeruginosa*-induced cases of unilateral panophthalmitis in turkeys (Nakamura and Abe 1987; Aziz and Barnes 2001), and *E. coli*-induced cases of

colisepticaemia-related panophthalmitis in chickens. The cellular nature of the panophthalmitis cases detected in chickens was described as being granulomatous (Gross 1957). Similarly, in the present study, based on the cellular characteristics determined by histopathological examination, panophthalmitis detected in the right eye of the penguin was defined as being pyogranulomatous. Granulomas are generally characterized by well-delineated focal lesions that develop in the event of the immune system's failure to eliminate infectious microorganisms. These lesions primarily contain mononuclear phagocytic cells, macrophages (histiocytes), epithelioid cells and multinucleated giant cells (Williams and Williams 1983). While bacterial and fungal granulomatous lesions associated with *Mycobacteria* spp., *Salmonella* spp., *Escherichia coli*, *Nocardia* spp., *Actinomyces* spp. and *Aspergillus* spp. have been detected in various systems of avian species, those associated with *Nocardia* spp. and *Actinomyces* spp. have been reported only rarely (Park and Jaensch 2009). In the current study, while Brown-Brenn staining of the ocular tissue sections prepared from the lesion demonstrated the presence of many Gram-positive filamentous pleomorphic bacteria, and Gram-negative coccobacilli and pleomorphic bacteria, GMS staining displayed the presence of branching filamentous bacteria surrounded by intense inflammatory cell infiltration, typically referred to as sulphur granules (Fig. S8). The culture method enabled the isolation of bacteria from the ocular lesion, which were determined as belonging to 5 different genera, and included *Pasteurellaceae*, *Corynebacterium ciconiae*, *Cardiobacteriaceae*, *Actinomyces* sp., and *Dermabacteraceae*. Although sulfur granules have been reported to occur in infections caused by agents such as *Nocardia* spp., *Staphylococcus* spp., and *Streptomyces* spp., (Wang et al. 2012), these agents could not be isolated in this study. In addition, filamentous structures characteristic of *Actinomyces* were detected in sulfur granules in the GMS staining. So these agents (*Nocardia* spp., *Staphylococcus* spp., and *Streptomyces* spp.) have been eliminated as possible aetiological agents based on these results. Also, no fungal growth having been observed upon mycological analysis, and the absence of acid-resistant bacteria having been confirmed by Ziehl-Neelsen staining in the histopathological and microbiological analyses eliminated the possibility of *Mycobacteria* spp. and *Aspergillus* spp. being involved in the aetiology of the granuloma. The bacteria, which were isolated by the culture method and identified as *Corynebacterium ciconiae*, *Actinomyces* sp. and *Dermabacteraceae* bacterium, all belong to the class Actinobacteria and cause granulomatous inflammation. Therefore, the granulomatous inflammation detected in the right eye of the penguin is considered most probably to have been caused by these particular organisms. There is only one previous study reporting granulomatous inflammation in penguins, which describes adenitis of the salt gland in blue penguins (*Eudyptula minor*) inhabiting New Zealand (Suepaul et al. 2010). Thus, to the authors' knowledge, this is the first study to report unilateral pyogranulomatous panophthalmitis in a Gentoo penguin chick. Many different bacteria, viruses, fungi and parasites have been isolated from cases of ocular surface disease in various avian species (Michelle Willis and Wilkie 1999). Given that ocular lesions occur as findings of either local or systemic infections, in general it is hard to determine whether the isolated organism is the primary cause of the lesion or not (Griggs 2019).

As is the case with mammalian cytokines, avian cytokines are also involved in the host immune response against infectious agents (Xu et al. 2019). MMPs are the key effectors and regulators of inflammation, wound healing, tissue regeneration and pathogenesis (Petznick et al. 2013). The correlation between certain inflammatory cytokines, including tumour necrosis factor-alpha (TNF α) and interleukin-1 (IL-1) (Shoshani et al. 2005; Cole et al. 2005; Kaiser and Stäheli, 2013) and MMP-9 (Shyu et al. 2019) has been demonstrated in

previous research on corneal diseases such as corneal ulcer and keratitis (Shoshani et al. 2005; Cole et al. 2005; Kaiser and Stäheli, 2013). Similar to previous reports in other avian species (Kaiser and Stäheli, 2013), it was also determined in the Gentoo penguin that pyogranulomatous panophthalmitis was associated with high level of MM-9 secretion and strong TNF α , IL-1 β , IL-6 and IL-4 immunoreactivity in response to the inflammatory stimulus (Fig. 3). In the present study, based on strong TNF α , IL-1 β , IL-6, IL-4 and MMP-9 immunopositivity having been detected in the inflammatory cells, it was considered that the indicated cytokines and enzyme could have accompanied the development of the granuloma in the eye diagnosed with mixed bacterial infection.

Staphylococcus spp., *Streptococcus* spp., *Clostridium* spp., *Erysipelothrix* spp., *Mycoplasma* spp., *Moraxella* spp., *Chlamydophila psittaci*, *Salmonella* spp., *Bordetella* spp., *Escherichia coli*, *Pseudomonas* spp., *Actinobacillus* spp., *Pasteurella* spp. and *Mycobacterium* spp. (Griggs, 2019) have been reported as ocular pathogens in avian species. Swinger et al. (2009) investigated the bacterial flora of the normal ocular surface as well as lacrimal secretion and intraocular pressure in the Humboldt penguin. These researchers reported to have isolated *Corynebacterium* spp., *Staphylococcus* spp., *Moraxella* spp. and *Pasteurella* spp. from the eyes of healthy captive penguins kept in freshwater and saltwater habitats at the Brookfield Zoo (Chicago, Illinois, ABD) and indicated that the most prevalent bacteria were *Corynebacterium* spp. and *Staphylococcus* spp. Similarly, Dupont et al. (1994) reported to have frequently isolated these two bacteria (*Corynebacterium* spp. and *Staphylococcus* spp.) alongside Gram-positive bacteria such as *Bacillus* spp. and *Streptococcus* spp., Gram-negative bacteria such as *Acinetobacter* spp., *Escherichia coli*, *Klebsiella oxytoca* and *Enterobacter* spp., and fungi such as *Aspergillus* and *Cladosporium* spp. from the eyes of healthy raptors.

The microorganisms isolated from the eyes of a Gentoo penguin chick in the present study differ from those reported to have been isolated from the eyes of mammals and exotic birds in previous studies (Silvanose et al. 2001; Cullen, 2003; Bonelli et al. 2014; Cakir et al. 2014; Sala et al. 2016). Furthermore, the microorganisms isolated in the present study have been determined to differ from the bacteria reported to have been detected in freshwater samples from the same location (Jara et al. 2020).

Today, the identification of microbial agents isolated from cases of infection by the culture method and molecular techniques is particularly significant for the establishment of treatment protocols (Miller et al. 2009). While the similarity of 16S rDNA gene sequences between two strains serves as a simple yet reliable criterion for the identification of novel strains, phylogenetic analysis has become the key to elucidate the evolutionary relationship between related taxa (Ludwig and Klenk 2001; Kim and Chun 2014).

Six bacteria, referred to as TAE3-ERU1/TAE3-ERU6, were isolated from samples taken from the right and left eyes of the penguin using the culture method. These bacteria underwent detailed investigation, including phenotypic analysis such as Gram staining, as well as molecular analysis and electron microscopic examination.

TAE3-ERU1, which was recovered from the right eye and identified as belonging to the family *Pasteurellaceae*, was 98.65% genetically similar to Bisgaard Taxon 14 according to the Genbank Database, and was found to be positioned between Bisgaard Taxon 14 and Bisgaard Taxon 32, based on phylogenetic analysis (Fig. S1). Based on EzBioCloud database, 16S rRNA similarity of TAE3-ERU1 was found to *Pasteurella testudines*

(94.22%). For describing of a novel genus has to be as an approximately 5- 6% divergence in 16S rRNA gene sequence from its closest genus in taxonomy studies (Qin et al. 2014). Due to the similarity value of TAE3-ERU1, we concluded that this isolate belonged to a novel genus in *Pasteurellaceae* family.

The family *Pasteurellaceae* includes 30 genera and 94 validly named species in current use (Bisgaard's taxa 2020; ICSP 2020; LPSN 2020a; Pasteurellacea 2020). This family also includes unidentified 55 taxa, known to have been isolated from various sources, which have been enumerated from 1 to 55 under the Bisgaard taxon (Bisgaard's taxa, 2020). Bisgaard Taxon 14 and Bisgaard Taxon 32 are known to be avian taxa (Christensen et al. 2003). Taxon 14 is reported to have been isolated from lesions of the upper respiratory tract in ducks, turkeys, pigeons, geese and peacocks (Bisgaard and Mutter, 1986; Bisgaard, 1993) and from cases of blepharoconjunctivitis in turkeys (Günther et al. 2006). Furthermore, Taxon 32 has been isolated from cases of pneumonia and conjunctivitis in pigeons and buzzards (Christensen et al. 2003).

TAE3-ERU2 was identified as a *Corynebacterium ciconiae* with 99.05%. The genus *Corynebacterium* comprises 125 species (LPSN, 2020b) of medical, veterinary or biotechnological importance, which have been isolated from various sources (Dorella et al. 2006). Members of this genus are not only part of the normal microbiota of the skin and mucosa in a variety of hosts, but can also be found in soil, water and the environment (Ott, 2018).

TAE3-ERU3 was found to show 94.70% similarity to the feline oral taxon of *Cardiobacterium* sp., according to the Genbank database. Also, Based on the EzBioCloud database, this isolate was close to *Pasteurella testudines* (94.22%) and *Rappaport israeli* (93.92%) and was determined a member of the *Cardiobacteriaceae* family. Therefore, based on phylogenetic analysis, it was determined that isolate TAE3-ERU3 could belong to a novel genus under the family *Cardiobacteriaceae* (Fig. S3). The family *Cardiobacteriaceae* includes three genera, namely, *Cardiobacterium*, *Dichelobacter* and *Suttonella*. In 2017, the species *Rappaport israeli* was isolated from human blood culture and was classified under a novel genus belonging to this family. However, no information is available on this novel genus in the LPSN database.

16S rRNA similarity of TAE3-ERU4, recovered from the right eye of the penguin, was found to *Actinomyces liubingyangii* with 97.40%. Phylogenetic analysis and similarity value of isolate are revealed isolate TAE3-ERU4 to be a novel *Actinomyces* species (Fig. S4). There are almost 29 validly named *Actinomyces* species. *Actinomyces* species are found on the mucosal surfaces of many animals, and access deeper tissues by means of trauma, surgical procedures, and foreign substances that disrupt the mucosal barrier (Park and Jaensch 2009). *Actinomyces* sp. has also been isolated from a case of canine ulcerative keratitis (Sherman et al. 2013).

16S rRNA similarity of TAE3-ERU5 was found to belong to *Dermabacteraceae*, and taxonomically, was determined to show 96.88% similarity to *Devriesea agamarum*. Classified under the Actinobacteria family, *Devriesea agamarum* has been reported to be associated with dermatitis and septicaemia in reptiles. Phylogenetic analysis demonstrated that isolate TAE3-ERU5 could belong to a novel genus in the *Dermabacteraceae* family, however, it is a different clade from *Devriesea*, *Helcobacillus*, and *Dermabacter* genus. (Fig. S5).

TAE3-ERU6 recovered from the healthy left eye of the penguin in the present study was identified as *Psychrobacter pygoscelis* on the basis that it showed 99.11% similarity to this species upon undergoing 16S rRNA sequence analysis. The number of newly described *Psychrobacter* species has increased in parallel with the increased number of discoveries related to polar and marine habitats. Today, the genus *Psychrobacter* includes 41 validly named species (Genus *Psychrobacter* 2020).

Isolates TAE3-ERU2, TAE3-ERU4 and TAE3-ERU5 (*Corynebacterium ciconiae*, *Actinomyces* sp., *Dermabacteraceae*), also recovered from the right eye, were determined to belong to the family Actinobacteria. While marine environments are known to be a rich source of Actinobacteria, bacteria in this family are reported to be capable of producing various active secondary metabolites (Yuan et al. 2014). Previous studies conducted in the Antarctic region (Li et al. 2010) and nearby Chile (Claverías et al. 2015; Undabarrena et al. 2016) have demonstrated the presence of Actinobacteria in marine sediment and soil (Lee et al. 2012). Claverías et al. (2015), who isolated Actinobacteria in Chile, investigated the potential of these bacteria producing antibacterial substances and reported that Actinobacteria in the marine sediment could be cultured and were richly diverse. Undabarrena et al. (2016) reported to have detected bacteria belonging to the families *Dermabacteraceae* and *Corynebacteriaceae*, under which the isolates TAE3-ERU5 and TAE3-ERU2 recovered in the present study were classified, among the Actinobacteria they isolated from marine sediment in Chile. Furthermore, other members of the family Actinobacteria, including *Arthrobacter* spp. *Brevibacterium* spp. and *Corynebacterium* spp. have also been reported to exist in the dry valleys of Antarctica (Cary et al. 2010).

All of the 5 isolates recovered in the present study were determined to be susceptible to each of the 7 antibiotics tested. Only the isolate belonging to the family *Dermabacteraceae* was found to be resistant to gentamicin. This high antibacterial susceptibility was attributed to penguins not being exposed to antibiotics. Several studies have been conducted to investigate the antibacterial susceptibility of various bacteria isolated from penguin faeces and water samples in the Arctic region (Bonnedahl et al. 2008; Miller et al. 2009; Rabbia et al. 2016; Cerdà-Cuellar et al. 2019; Jara et al. 2020). Although literature reports have been published on bacterial isolation from cases of infection in penguins in the Antarctic region (Nievas et al. 2007), no information is available on the antibacterial susceptibility of these isolates.

Today, only very few geographical regions, such as the Antarctic, are free from direct and indirect contact with human populations and domestic animals (Barbosa and Palacios 2009). In a recent study, in which bacteria were isolated from freshwater samples pertaining to different regions of the Antarctic Peninsula that were either subjected to or free from human contact and effect, Jara et al. (2020) assessed the antibacterial susceptibility of these isolates. Accordingly, these researchers reported that the number of antibiotic-resistant bacteria and antibiotic resistance genes were higher in the regions that were affected by humans and wildlife, compared to the regions that were far from human and animal populations.

In the present study, bacteria of 5 different genera were isolated from the right eye of the penguin. Thus, it was not possible to identify the primary causative agent of the pyogranulomatous inflammation in the right eye. Cytological and histopathological analyses demonstrated pyogranuloma as a characteristic finding of *Actinomyces* sp. infection, and the extra- and intracellular presence of various other Gram-positive and Gram-negative bacteria was also determined in the ocular lesion.

Conclusions

The concurrent isolation of 5 different bacteria from the case of unilateral panophthalmitis in the right eye of the penguin is considered to be very interesting. In the present study, the sample taken from the lesion in the right eye was directly inoculated onto solid medium, and based on the assessment of macroscopic and microscopic morphology, the intense growth of 5 different bacterial colonies was observed. Different types of colonies having been detected was attributed to direct inoculation having been performed with no prior enrichment. Four different putative novel bacteria, bearing the potential of being new genera, species or taxa, having been isolated from the lesion in the right eye, and *Psychrobacter pygoscelis* having been isolated from the left eye of the animal are considered to be indicative of the rich bacterial diversity in the habitat of the penguin. The severity of the lesion was considered to be related also to the age of the animal. Studies that include further molecular analysis and phenotypic tests for the identification of our isolates at the species level continue.

Declarations

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Author Contributions

Latife Cakir Bayram: designed the research and collected the samples from the field; **Secil Abay and Fuat Aydin:** performed the bacteriological analysis of the samples and PCR for the identification of bacteria and 16S rRNA gene sequencing and analysed antibacterial susceptibility of the isolates; **Tolga Guvenc and Gorkem Ekebas:** performed immunohistochemical staining of cytopsin preparations and tissue sections; **Latife Cakir Bayram:** prepared cytopsin slides, stained tissue sections and cytopsin smears with special staining methods and interpreted the results; **Izzet Burcin Saticioglu:** prepared and evaluated the phylogenetic trees; **Latife Cakir Bayram, Secil Abay and Fuat Aydin:** discussed the results and wrote the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Consent for publication

Not applicable

Ethics approval

Approvals regarding entrance to the specially protected area of the Antarctic (Antarctic Specially Protected Area, ASPA, No: 150, Permit N°- 21- 2019) and for collecting eye samples from the penguins (Antarctic Specially Protected Area, ASPA, No: 150, Permit N°- 07- 2019) were provided by the Turkish Antarctic Program.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. The phenotypic and physiological characteristics of the isolates

Isolates	TAE3- ERU1	TAE3-ERU2	TAE3- ERU3	TAE3- ERU4	TAE3-ERU5	TAE3-ERU6
Characteristics						
Gram staining	Negative	Positive	Negative	Positive	Positive	Negative
Morphology	Coccobacilli	Bacilli and coryneform	Bacilli	Bacilli	Cocobacilli and bacilli	Cocobacilli and bacilli
Motility	-	-	-	-	-	-
Growth in NaCl (w/v)						
0	+	+	-	-	+	-
2%	+	+	+	+	+	+
5%	+	+	+	+	+	+
10%	-	+	+	-	-	-
Growth at						
4 °C	+	++	+	++	++	+
22 °C	+	+	+	+	+	+
37 °C						
Aerobic	+	+	+	+	+	+
Microaerobic	+	+	+	+	+	+
Anaerob	+	+	-	+	+	-
Mac Conkey agar	+	-	-	-	-	-
42 °C	+	+	-	+	+	++
Activity of						
Urease	-	-	-	-	-	+
Oxidase	-	-	+	-	-	+
Catalase	+	+	+	+	+	+
Esculin	+ ^v	-	-	-	-	-
Utilization of specifis sugars						
Mannitol	-	-	-	-	-	-
Xylose	-	-	-	-	+	-

Arabitol	-	-	-	-	-	-
Ribose	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
Tagatose	-	-	-	-	-	-

*: weakly, v: variable

Table 2. Information on the GenBank-registered bacteria, to which the 16S rRNA gene sequence results of the isolates showed similarity, and the rates of similarity

Isolates	Accession Number	BLAST results		EZBio Cloud		GenBank informations of the isolates in this study
		Species matched	Per. ident	Species matched	Per. ident	
TAE3-ERU1	MT444110.1	Bisgaard taxon 14 strain	98.65	<i>Pasteurella testudinis</i>	94.22	<i>Pasteurellaceae</i> bacterium
TAE3-ERU2	MT444111.1	<i>Corynebacterium ciconiae</i>	98.76	<i>Corynebacterium ciconiae</i>	99.05	<i>Corynebacterium ciconiae</i>
TAE3-ERU3	MT444112.1	<i>Cardiobacterium</i> sp. feline oral taxon	94.70	<i>Pasteurella testudines</i>	94.22	<i>Cardiobacteriaceae</i> bacterium,
				<i>Rappaport israeli</i>	93.92	
TAE3-ERU4	MT444113.1	<i>Actinomyces liubingyangii</i>	97.19	<i>Actinomyces liubingyangii</i>	97.40	<i>Actinomyces</i> sp.
TAE3-ERU5	MT444114.1	<i>Devriesea agamarum</i>	97.14	<i>Devriesea agamarum</i>	96.88	<i>Dermabacteraceae</i> bacterium
TAE3-ERU6	MT674807.1	<i>Psychrobacter pygoscelis</i>	99.03	<i>Psychrobacter pygoscelis</i>	99.11	<i>Psychrobacter pygoscelis</i>

Figures

Figure 1

The region in ASPA 150 Ardley Island, where the ocular samples were taken from a Gentoo penguin (a-d). The orange box shows the continental position of the Antarctic Peninsula (a). The orange box shows the location of King George Island (b). The red arrowhead indicates the material collection site on Ardley Island; scale bar 1000 m (c). The research access area and nearby stations are seen in the satellite image (d). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Evaluation of eye lesion on Gentoo penguin (*Pygoscelis papua*) chick A) The right eye of a Gentoo penguin chick. A reddish-orange exophytic tissue obscured the view of the cornea. B) The corneal surface of the Gentoo penguin was sampled for bacterial culture.

Figure 3

Histopathology of the exophytic tissue in the right eye of the penguin (A–AB) A) Pyogranuloma in the anterior chamber of the eye, including the cornea and iris: Necrosis (n), infiltration of numerous macrophages and heterophils, and fewer lymphocytes (*) characterized by giant cells surrounding the central necrotic region (arrowheads). B) Numerous infiltrating cells consisting mainly of mononuclear cells, imaged at a higher magnification as shown by a star (*) in Fig. A. C) Fibrin, bacteria, and necrotic inflammatory cells (yellow boxed area) and haemorrhage. Inset: Higher magnification view of the yellow boxed area. D) Higher

magnification view of the necrotic area (n) in Figure (A). Necrotic debris and distended macrophages containing bacteria (yellow boxed area). E) Pyogranulomatous nodule surrounded by infiltrates of lymphocytes and plasma cells. Infiltration of the necrotic area with heterophils and macrophages containing numerous bacteria (boxed area). F) Brown-Brenn staining of Figure (A). Severe pyogranulomatous inflammation with inflammatory cell infiltration composed of lymphocytes, plasma cells, and histiocytes (*). Macrophages with bizarre nuclei (dark blue boxed area). An abundance of Gram-positive bacteria in and around an area of necrosis (yellow boxed area, Inset: Higher magnification of the yellow boxed area). The image shows Gram-positive bacteria of different morphology, observed as many intracellular and extracellular clumps within the exudate. G) Higher magnification of the dark blue boxed area in Figure (F). An abundance of Gram-positive bacteria found in and around an area of necrosis and macrophages with bizarre nuclei (arrow). H) Higher magnification of the star (*) in Fig. B. Corneal epithelium infiltrated with reactive macrophages and lymphocytes. I) Higher magnification of the star (*) in Figure F. Macrophages with bizarre nuclei (arrow), and many degenerate neutrophils and multinucleated giant cells with several cocci within their cytoplasm. J) Brown-Brenn staining of Fig. 1E. Ocular manifestations of the anterior segment. Dense mononuclear infiltration. Necrotic cell debris with multiple Gram-positive and Gram-negative bacteria (boxed area). K) An abundance of Gram-positive and Gram-negative bacteria within and around an area of necrosis. Inset: Higher magnification. L) Pyogranulomatous inflammation composed mainly of reactive macrophages (bizarre cells) (red boxed area), heterophils, and fewer lymphocytes. M) Higher magnification of the red boxed area in Figure L. Gram-positive (red arrow) and Gram-negative bacteria (blue arrow) in the cytoplasm of macrophages. N) Numerous Gram-positive bacilli in the area of necrosis. O) Higher magnification of the Brown-Brenn staining of the yellow boxed area in Figure D. Typical granuloma. Numerous bacteria of different morphology in the parenchyma of the anterior camera. Filamentous and branching Gram-positive bacteria. P) Higher magnification of the yellow boxed area in Fig. J. Gram-positive and Gram-negative cocci, coccobacilli and filamentous bacilli. Q) Multiple granulomas with central necrosis (one is indicated by a yellow star). Granulomatous inflammation consisting of macrophages, lymphocytes, and fewer heterophils (one is indicated by a white star). R-X) Immunohistochemical staining of pro-inflammatory cytokines in the conjunctival epithelium of the right eye with panophthalmitis. R, S) Demonstration of IL-1 β by IHC staining in the granuloma cells. R) IHC staining of Figure A. Langhans giant cells with positive immunoreactivity (one is indicated by a yellow arrow), and many lymphocytes, macrophages and epithelioid cells with negative immunoreactivity (*) to IL-1 β . S) Langhans giant cells strongly immunostained for IL-1 β . T) IL-4 expression in the granuloma cells. U-V) Macrophages with positive immunoreactivity for IL-6 (shown by yellow and black arrows). W) Langhans giant cells (yellow arrows) and conjunctival goblet cells (one is shown by the blue arrow) with positive immunoreactivity for IL-6. Y, Z) MMP-9 expression in the corneal epithelium (yellow arrows). X) Blue arrows indicate stromal keratocytes with strong MMP-9 expression. AA-AB) Strong immunoreactivity of the choroid and sclera for TNF- α . Bars of main plate: 20 μ m (P, R-U, W, X, Z, AB), 50 μ m (B, H, M, N), 100 μ m (A, D, E, G, J, O, V, Y, AA), and 200 μ m (C, F, I, K, L, Q). Bars of insets: 50 μ m (C, K), 100 μ m (F). Stains of main plate: Haematoxylin and eosin (A-E), Brown-Brenn Gram (F-Q), immunoperoxidase-3,3'-diaminobenzidine (DAB chromogen and counterstained with haematoxylin) (R-AB). Stains of insets: Haematoxylin and eosin (C) and Brown-Brenn Gram (F, K).

Figure 4

Morphological analysis of the bacterial isolates by light microscope (panels on the left-hand side, Gram staining) Transmission electron microscope (panels on the middle) and Scanning electron microscopy SEM (panels on the right-hand side) Standard cell morphology of TAE3- ERU1 (*Pasteurellaceae* bacterium, A-C), TAE3- ERU2 (*Corynebacterium ciconiae*, D-F), TAE3- ERU3 (*Cardiobacteriaceae* bacterium, G-I), TAE3- ERU4 (*Actinomyces* sp., J-L), TAE3- ERU5 (*Dermabacteraceae* bacterium, M-O) and TAE3- ERU6 (*Psychrobacter pygoscelsis*, P-S) Bar: 10 μ m (A,D,G,J,M,P), 1 μ m (E,C,F,S), 100nm (I), 200 nm (B,H,K,L,R,O)