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Rothia aeria and *Rothia dentocariosa* as biofilm builders in infective endocarditis

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ABSTRACT

Background: *Rothia* sp. are Gram-positive bacteria in the class of *Actinobacteria* that are part of the physiological oral flora. In rare cases, *Rothia aeria* and *Rothia dentocariosa* can cause infective endocarditis (IE). The biofilm potential of *Rothia* in endocarditis is unknown.
Methods: Specimen from two cases of *Rothia* endocarditis were obtained during cardiac surgery. One of the patients suffered mitral valve IE from *Rothia aeria*. In the other case, IE of a prosthetic pulmonary valve was caused by *Rothia dentocariosa*. Fluorescence in situ hybridization (FISH) was used for visualization of microorganisms within heart valve tissues in combination with PCR and sequencing (FISHseq).
Results: The two heart valve specimens featured mature biofilms of bacteria that were identified by FISHseq as *Rothia aeria* and *Rothia dentocariosa,* respectively. FISH showed in situ biofilms of both microorganisms that feature distinct phenotypes for the first time ex vivo. Both of our reported cases were treated successfully by heart valve surgery and antibiotic therapy using beta-lactam antibiotics.
Conclusion: The biofilm potential of *Rothia* sp. must be taken into account. The awareness of *Rothia aeria* and *Rothia dentocariosa* as rare but relevant pathogens for infective endocarditis must be raised. Use of biofilm-effective antibiotics in *Rothia* IE should be discussed.

1. Introduction

Infective endocarditis (IE) is an inflammation of the endocardium and has a high mortality rate as well as severe complications (Habib et al., 2015). In most cases, IE is caused by bacterial pathogens, rarely by fungi. Although the microbiological diagnosis is essentially based on positive blood cultures, pathogen detection is not successful in 2.5 %–31 % of patients (Fournier et al., 2010). In cases of blood culture negative endocarditis, molecular biological methods such as nucleic acid amplification techniques (NAT) and fluorescence in situ hybridization (FISH) can help to identify the causative organism in situ (Mallmann et al., 2010). FISH can also provide information about the activity of the endocarditis pathogens and the organization of microorganisms in possible biofilms (Yaban et al., 2020).

In 80–90 % of endocarditis cases, the causative organism is one of the Gram-positive cocci *Staphylococcus* sp., *Streptococcus* sp., or *Enterococcus* sp. (Cahill and Prendergast, 2016). Two of the rather rare bacterial species associated with IE are *Rothia aeria* and *Rothia dentocariosa*. *Rothia* sp. are Gram-positive, non-acid-fast bacteria in the class of *Actinobacteria*. After the genus *Rothia* was proposed in 1967 with *Rothia dentocariosa* being one of the already known species (Georg and Brown, 1967), the species *Rothia aeria* was initially described in 2004 after

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isolation from the air inside a Russian space station (Li et al., 2004). In the past nomenclature, *Rothia aeria* was referred to as *Rothia dentocariosa* genomovar II (Kronvall et al., 1998). Both *Rothia aeria* and *Rothia dentocariosa* are part of the physiological flora of the oral cavity making up 0.8 % and 1.3 % of the human oral microbiome (Tsuzukibashi et al., 2017). *Rothia aeria* is rarely detected in clinical material, but can be of etiological relevance: Bacteremia, septic arthritis, ovarian tube abscess, cervical abscesses, infections of the respiratory tract and endocarditis have been described (Michon et al., 2010; Verrall et al., 2010; Mahobia et al., 2013; Falcone et al., 2012; Taira and Aoki, 2019; Ramanan et al., 2014; Collarino et al., 2016; Crowe et al., 2014; Thiyagarajan et al., 2013; Tarumoto et al., 2012; Nicodemo et al., 2014; Kim et al., 2014; Hiraiwa and Izumi, 2013). IE caused by *Rothia aeria* is a very rare condition since to date, there are only six reported cases worldwide. Most of these cases had severe complications such as central nervous system embolisms or hemorrhages (Crowe et al., 2014; Thiyagarajan et al., 2013; Tarumoto et al., 2012; Hiraiwa and Izumi, 2013). Concerning the treatment of *Rothia aeria* endocarditis, the antibiotic regime carried out in the various case reports is inconsistent. IE related to *Rothia dentocariosa*, however, is a somewhat more common constellation since its first reported case appeared in 1978 (Pape, 1979). As of July 2020, there are 22 reported cases of *Rothia dentocariosa* endocarditis worldwide (Willner et al., 2019). So far, no images of in vivo grown *Rothia* sp. in tissue are available.

Although complete gene sequencing of *Rothia aeria* has already been successful, its virulence factors remain unknown (Nambu et al., 2016). In general, IE can be associated with formation of microbial biofilms in the heart valves being recalcitrant to antibiotic treatment (Hall-Stoodley et al., 2012). There are no data thus far, on whether *Rothia* species form biofilms in vivo in tissue. In order to characterize a potential property as a biofilm builder, we analysed *Rothia aeria* and *Rothia dentocariosa* IE by FISHseq to identify the pathogens in sample material from two cases of IE.

2. Methods

2.1. Specimen processing and fluorescence in situ hybridization (FISH)

Samples of valve material from two cases of IE and in vitro grown cultures of the *Rothia dentocariosa* strain were analyzed. The heart valve specimen and cultured strain were fixed with FISHopt® (MoKi Analytics, Berlin, Germany) at 4 °C for 24 h and subsequently embedded in cold polymerizing resin and sectioned as described before (Moter et al., 1998). Hybridization was carried out as described before (Mallmann et al., 2010) with the oligonucleotide probes EUB338 and NONEUB338 (Biomers, Ulm, Germany) and the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI). The probe EUB338 is complementary to part of the 16S rRNA gene conserved in most microorganisms of the domain bacteria and was used to screen for bacterial colonization (Amann et al., 1990). The probe NONEUB338, which is the antisense probe of EUB338, was used to exclude nonspecific probe binding (Gescher et al., 2008a). Genus- or species-specific FISH-probes for identification of streptococci, enterococci, staphylococci, *Staphylococcus aureus*, *Tropheryma whipplei*, *Bartonella quintana*, *Coxiella burnetii*, or *Granulicatella* were used as described (Gescher et al., 2008a; Geissdörfer et al., 2012; Aistleitner et al., 2018; Gescher et al., 2008b). After incubation for 2 h in a dark humid chamber at 50 °C, slides were rinsed with water, air-dried, and mounted with mounting medium.

For microscopy, an epifluorescence microscope (AxioImagerZ2; Carl Zeiss, Jena, Germany) equipped with narrow band filter sets (AHF-Analysentechnik, Tübingen, Germany) was used.

2.2. 16S rRNA gene amplification and sequencing

DNA was extracted from sections of the embedded heart valve tissues. The samples were submitted to pan-bacterial amplification of part of the 16S rRNA gene using the primers TPU1 and RTU3, as described earlier (Moter et al., 1998). Amplicons were sequenced using a commercial sequencing facility (LGC Genomics, Berlin, Germany) and compared with all currently available sequences from the public databases (EMBL and GenBank) using the commercial SmartGene program (SmartGene, Lausanne, Switzerland).

2.3. Ethics statement

The analysis was performed in accordance with the ethical guidelines of the 1964 Declaration of Helsinki and was approved by the Ethics committee of the Charité – Universitätsmedizin Berlin (E82/018/18). As the study was performed as part of the routine diagnostic work-up, the need for informed consent was waived according to the ethics committee approval.

3. Patient description

The first patient was an 18-year-old woman who was admitted to the Charité - Universitätsmedizin Berlin for surgical treatment of a severe mitral regurgitation due to IE. In addition to dietary managed phenylketonuria, there was no other known medical condition. The initial consultation was made via emergency unit for flu symptoms, temperature of 39.0 °C and limited physical resilience that had been present for 4 weeks. Indurations on the hands and feet had previously been diagnosed by the family doctor as hand-foot-and-mouth disease, but were probably Janeway-Lesions from a retrospective point of view. A computed tomography (CT) scan of the thorax showed ground glass opacity in the right lower lobe as well as thickened inter- and intralobular septa compatible with interstitial viral pneumonia. Because the patient presented during the pandemic of coronavirus disease 2019 (Covid-19), a combined oro-nasopharyngeal swab was obtained to be tested for SARS-CoV-2 RNA which returned negative. Nevertheless, a superinfection based on viral pneumonia would be a conceivable entry point for endocarditis. Expert examination from the department for oral and maxillofacial surgery excluded a possible dental focus. On examination, the patient showed a tachycardia up to 110 bpm and a 4/6 holosystolic murmur. Echocardiography revealed a 6 × 6 mm floating structure on the anterior mitral leaflet, as well as severe mitral regurgitation due to a substance defect close to the posteromedial commissure. After three pairs of peripheral blood cultures were obtained, a guideline-based empiric antibiotic therapy with ampicillin, flucloxacillin and gentamicin was initiated.

Minimally invasive mitral valve reconstruction was performed using a right anterolateral minithoracotomy. Intraoperative findings showed ruptured A3 and P3 tendinous cords causing the posteromedial commissure to prolapse. Vegetations about 5 mm in size at the end of the torn cords were preserved for microbiological examination. Implantation of four adjustable neochords (Gore-Tex™, GORE-TEX; W. L. Gore & Associates Inc., Newark, DE, USA) in the A3/P3 segments, posteromedial commissural plasty and implantation of a 30 mm annuloplasty ring (CG Future™ Ring, Medtronic Inc., Minneapolis, MN, USA) was performed. Postprocedural echocardiography demonstrated a competent mitral valve without residual regurgitation. The postoperative course was uncomplicated.

After 33 h of incubation, all three preoperative blood cultures showed growth of Gram-positive coryneform rods that were identified as *Rothia aeria*. The result of the susceptibility testing is shown in Table 1. Due to resistance to gentamicin, antibiotic therapy was switched to high dose intravenous ampicillin (2 g, 6x/day). The FISHseq diagnostics performed on the intraoperative samples confirmed a highly active endocarditis with extensive biofilm components by *Rothia aeria* (as seen in Fig. 1). In the intraoperative material, *Rothia aeria* was still detectable in microbiological culture despite 4 days of intravenous antibiotic treatment. Therefore, a duration of antibiotic therapy of 6 weeks starting from the date of the operation was suggested. After three weeks of

Table 1
Susceptibility testing for *Rothia aeria* and *Rothia dentocariosa* detected in the presented cases.

	Rothia aeria		*Rothia dentocariosa*		
antibiotics	susceptibility	MIC	susceptibility	MIC	breakpoint
Penicillin	S	0.008/0.016*	S	0.003	0.25
Ampicillin	S	0.016	–	–	2
Amoxicillin + clavulanic acid	S	0.016	–	–	2
Piperacillin + Tazobactam	S	0.047	–	–	4
Cefotaxime	–	–	S	0.032	1
Meropenem	S	0.500	–	–	2
Gentamicin	R	1.500	R	2	0.5
Moxifloxacin	S	0.25	–	–	0.25
Clindamycin	S	0.25	–	–	–
Vancomycin	S	1.	S	1	–
Rifampicin	S	0.016	S	0.004	–
Linezolid	S	0.5	–	–	2
Tigecycline	S	0.125	–	–	0.5

Note. There are no specific EUCAST limits for interpreting sensitivity for this species. Assumed cut offs are based on *Corynebacterium* spp. Values are given in mg/L. * = tested in blood culture and sample from surgery, S = susceptible, R = resistant, Breakpoint = EUCAST PK-PD limits, MIC = minimum inhibitory concentration.

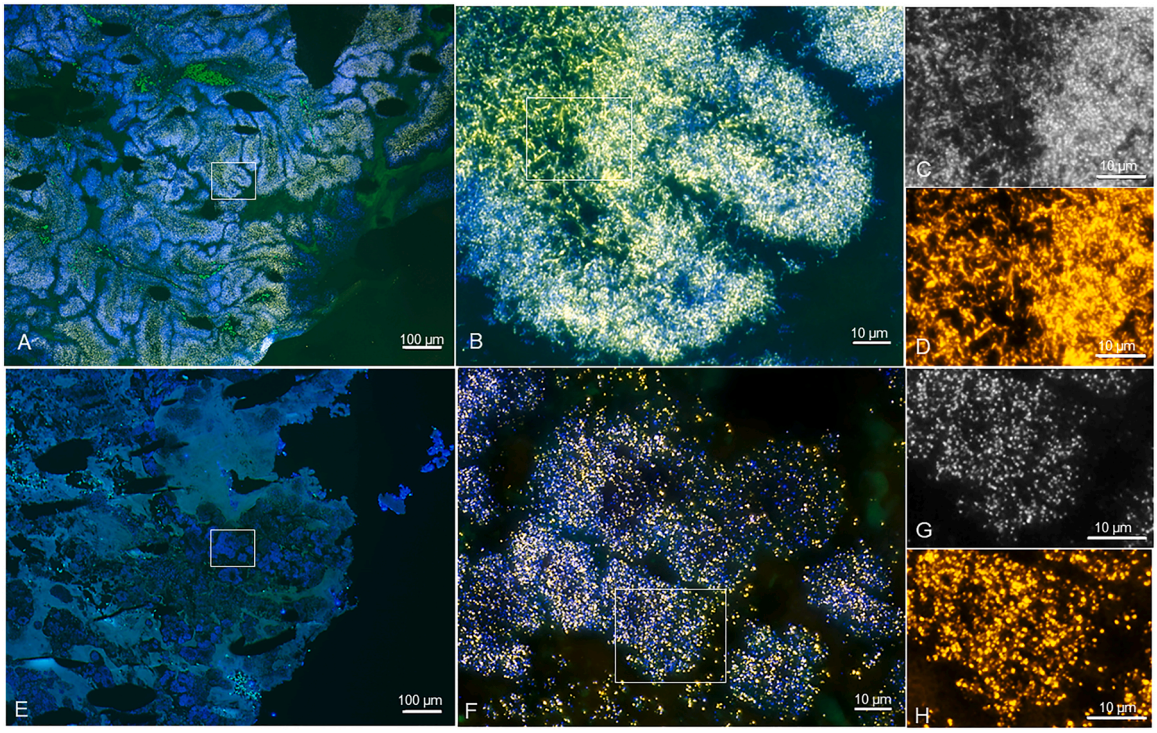

Fig. 1. Detection of biofilms by FISHseq in the heart valve samples by *Rothia aeria* and *Rothia dentocariosa*.
FISH revealed extensive bacterial biofilms in the heart valve tissues from *Rothia aeria* IE (A–D) and *Rothia dentocariosa* IE (E–H). Sections were hybridized with the probes EUB338 (Cy3-labeled, orange) and NONEUB (Cy5-labeled, no signals visible), and stained with the nucleic acid stain DAPI (blue). Tissue background appears in green. We found impressive bacterial biofilms that were to a large part FISH-positive (orange) besides cells visible with the nucleic acid stain DAPI only (blue). The strong FISH signal corresponds to a high ribosome content and indicates bacterial activity at the time of surgery. **A** and **E**: Overview of the heart valve tissue specimen from patient 1 and 2, respectively, showing impressive bacterial biofilms (DAPI, blue) versus the tissue background (green). While the majority of the sample in patient 1 (A) consisted of bacteria in an undulating biofilm, patient 2 (E) showed extensive biofilm fields within a largely destroyed heart valve. **B** and **F**: Magnification of the insets marked in A and E, respectively, show distinct cells positive for the probe EUB338 (orange) in the overlay with the DAPI channel (blue). **C** and **G**: Magnification of the insets marked in B and F, respectively, show the separate DAPI channel in black and white. **D** and **H**: The same field of view as C and G, respectively, show the separate channel of the probe EUB338 (orange). Note the discriminative appearance of *Rothia aeria* as more rod-like as compared to *Rothia dentocariosa*, which is more coccoid.

treatment, antibiotic therapy was switched to ceftriaxone (2 g per day) and a peripherally inserted central catheter (PICC line) was implanted to allow antibiotic treatment to be continued on an outpatient basis within a specialized outpatient parenteral antimicrobial therapy (OPAT) program. After a total antibiotic therapy duration of 6 weeks, the antibiotic therapy was completed. The patient no longer showed any clinical signs of infection. Leukocytes were preoperatively measured at a level of 13.1/nL, rose to a maximum of 20.5/nL postoperatively and were 5.0/nL one week after the end of antibiotic therapy. C-reactive protein test was at a level of 2.1 mg/l at the same follow-up. Successful eradication of the pathogen, which caused IE, is therefore assumed.

The second patient was a 46-year-old man who underwent Ross-procedure due to severe aortic valve insufficiency. The pulmonary valve replacement was carried out using the Matrix P Plus® prosthesis (AutoTissue GmbH, Berlin, Germany), which is a decellularized porcine xenograft. Three years after the surgery, the patient presented to his

cardiologist with symptoms of a cold that had existed for 8 weeks, as well as limited physical resilience and stress dyspnoea. Echocardiography revealed a combined lesion of the pulmonary valve prosthesis with severe stenosis and moderate insufficiency, as well as a 14 × 9 mm adherent floating structure. The patient was admitted to the Charité - Universitätsmedizin Berlin for redo surgical treatment. An empiric antibiotic therapy with vancomycin, rifampicin and gentamicin was initiated. During redo surgery, suspected endocarditis was confirmed by the presence of two adhering vegetations. The infected pulmonary valve prosthesis was replaced by a new 25 mm Matrix P Plus® and preserved for microbiological examination. The postoperative clinical course was uncomplicated.

Blood cultures obtained before the initiation of antibiotic therapy showed growth of coccoid/rod-shaped Gram-positive bacteria in the aerobic bottles after two days of incubation that were identified as *Rothia dentocariosa*. The associated result of the susceptibility pattern is shown in Table 1. The FISHseq diagnostics performed on the valve material confirmed the pathogen to be *Rothia dentocariosa* causing a highly active endocarditis with extensive biofilms (as seen in Fig. 1). After only two weeks of intravenous antibiotic therapy, the regimen was switched to oral administration of Penicillin V. The patient was discharged home under careful monitoring and did not show any clinical signs of infection. The antibiotic therapy was completed after a total duration of four weeks. All follow-up blood cultures obtained after the initiation of antibiotic therapy showed no growth in the long-term incubation. The valve material obtained intraoperatively after two days of empiric antibiotic treatment also showed no bacterial growth after 14 days of incubation. Leukocytes were 16.4/nL before the start of treatment, rose to a postoperative maximum of 27.2/nL and were 6.2/nL when discharged. C-reactive protein test was at a level of 5.4 mg/l at discharge. However, the patient was lost to follow-up and there is no information on the clinical course after completion of antibiotic therapy.

4. Results

Both heart valve specimen were fixated intraoperatively and analyzed by FISHseq. For FISH, we screened with the pan-bacterial probe EUB338 for bacterial colonization including yet uncultivated or fastidious bacterial species. We found extensive structured biofilms, which were to a large part FISH-positive, in both cases. None of the specific FISH probes for the species most commonly associated with IE identified the EUB338-positive bacteria. Therefore, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and the rare IE pathogens *Granulicatella*, *Tropheryma whipplei*, *Coxiella burnetii* and *Bartonella quintana* were excluded as infectious agents.

For sequencing, DNA was isolated from consecutive sections of embedded heart valve tissue and submitted to pan-bacterial PCR amplification. Sequencing of the PCR products resulted for the first patient in a 433-base pair fragment with the highest homology (100 % identity) to the *Rothia aeria* 16S rRNA gene (centroid database of SmartGene, Basel, Switzerland, accession number AB753461). Similarly, for the second patient, a 480-base pair fragment was obtained with the highest homology (100 % identity) to the *Rothia dentocariosa* ATCC 19731 16S rRNA gene (centroid database of SmartGene, accession number CP002280). Both obtained sequences differed with seven mismatches from each other.

Fig. 1 shows the results of the microscopy performed on both heart valve samples after processing via FISHseq. The microscopic analysis revealed extensive biofilms in both cases. Biofilms are structured bacterial communities, which can be visualized by FISH: the fluorescence signal intensity of FISH correlates with the bacterial ribosome content and therefore visualizes the spatial organization of the bacterial activity (Stewart et al., 2016; Sutrave et al., 2019). In the *Rothia aeria* case, the sample consists of parts of the mitral subvalvular apparatus. As can be seen in Fig. 1A, the entire organic matter of the sample was formed by garland-like structures, which consisted almost entirely of bacteria. In contrast, a largely destroyed heart valve is visible in patient 2, which is infiltrated by extensive biofilms (Fig. 1E). Both cases presented here display areas with a higher ribosome content correlated to more active areas, and areas, which are mainly DAPI-positive and FISH-negative, correlated to less active regions within the biofilm. Whereas *Rothia aeria* appeared mainly as long rods (Fig. 1D), *Rothia dentocariosa* predominantly presented as coccoid rods (Fig. 1H). Fig. 1 shows the first in vivo grown biofilms from *Rothia aeria* and *Rothia dentocariosa*.

To analyse the morphology and biofilm potential of *Rothia dentocariosa* in vitro, we fixated the strain grown in liquid culture (Fig. 2B and C), a colony from an agar plate (Fig. 2A, C–F), a nascent 2-days old biofilm (Fig. 2H–K) and a mature 7-days old biofilm (Fig. 2M–P) grown on a polyurethane chip in a shaking culture to FISH analysis. The 7-days old biofilm culture showed indeed a mature biofilm, which was quite similar to those in the ex vivo heart valve. Most bacteria had a coccoid morphotype whereas in the liquid culture and young biofilm also long rods were visible (Fig. 2C, K, L).

5. Discussion

In rare cases, IE can be caused by *Rothia aeria* or *Rothia dentocariosa*. To our knowledge, our reported case of *Rothia aeria* IE is the seventh reported case worldwide and the first one in Germany. The case of *Rothia dentocariosa* IE represents the 23rd reported case worldwide. Both cases had a fortunate clinical course without thromboembolic complications. In the *Rothia aeria* case, the entry point for the bacteria ultimately remains unclear, since according to the dental examination, there was no sign of an odontogenic infection. In addition to the oral flora, *Rothia aeria* is also present in the flora of the upper respiratory tract (Ramanan et al., 2014). As suggested by the findings of the CT, a pre-existing viral pneumonia might have predisposed for a bacterial superinfection. In the *Rothia dentocariosa* case, an odontogenic source of infection is likely and the patient was advised to undergo prompt restorative care by dental professionals.

In our *Rothia aeria* case, microbiological culture of the sample obtained intraoperatively still showed bacterial growth after four days of antibiotic treatment. The strong FISH signal also indicated active bacterial growth at the time of surgery although antibiotic treatment had already started. In both *Rothia* sp. cases, FISHseq revealed impressive in vivo grown biofilms in the heart samples confirming their biofilm building capacity. As far as we know, these are first cases showing the biofilm potential of *Rothia aeria* and Rothia *dentocariosa* in vivo. However, the biofilm architecture differed between patients. The *Rothia aeria* case imposed by garland-like structures consisting of highly active bacteria, which is compatible with a highly active, rapidly developed endocarditis. In the Rothia *dentocariosa* case, the largely destroyed heart valve was infiltrated by biofilms, again with highly active bacteria. The demonstrated biofilm-forming potential indicates the relevance of surgical treatment for control of infection and successful bacterial eradication.

Since images of *Rothia* spp. are scarce, we provide with Fig. 2 a comprehensive overview of the morphology of *Rothia dentocariosa* from different growth forms and stages. We found that the morphology of the bacterium may vary substantially depending on the culture conditions. Therefore, the Gram-stain of *Rothia* spp. may be difficult to interpret in the clinical setting where the growth condition of the bacteria must be unknown.

In our *Rothia aeria* case, antibiotic treatment was carried out using high dose intravenous penicillin for three weeks, changing to further three weeks of intravenous ceftriaxone. While beta-lactam antibiotics were administered in all previously reported cases, the exact treatment regimens differ significantly. In three of the reported cases, treatment was switched to a monotherapy using an amino-penicillin or ceftriaxone after initial empiric therapy (Collarino et al., 2016; Nicodemo et al., 2014; Kim et al., 2014). In the case reported by Thiyagarajan et al., a combination of ceftriaxone and rifampicin was administered

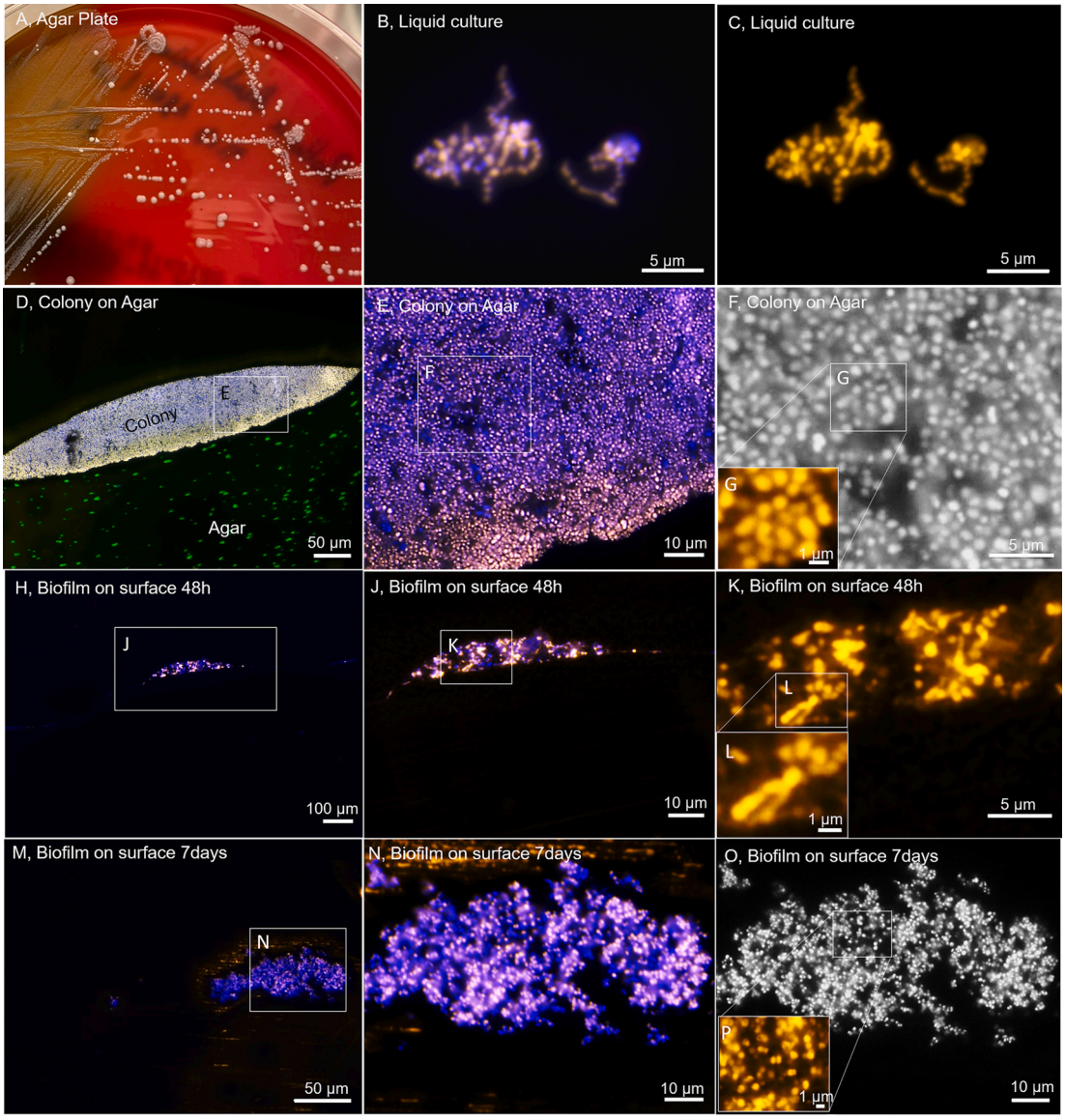

Fig. 2. In vitro morphology of *Rothia dentocariosa*.

We assessed the in vitro morphology of *Rothia dentocariosa* macroscopically on an Agar plate, in liquid culture, and in in vitro grown biofilms. Our aim was to address the question whether the morphology of the bacteria (rods or cocci) might help the clinical microbiologists with the identification of this pathogen from blood or tissue culture.

The **upper panel** (**A**–**C**) shows on the left the growth of *Rothia dentocariosa* colonies on a blood Agar plate (A). Note the characteristic concentric terrace shaped appearance in particular in larger colonies. B and C show *Rothia dentocariosa* from liquid culture by Fluorescence in situ hybridization (FISH). Note the varying appearance of the bacteria as coccoid and rod-like. B shows an overlay of the DAPI-channel (blue) staining the nucleic acids, and the Cy3-channel (orange, EUB338 probe). C shows the separate Cy3-channel (orange, EUB338 probe).

The **upper middle panel** (**D**–**G**) shows a *Rothia dentocariosa* colony on agar embedded in methacrylate, sectioned and visualized by FISH. In the overview (D), the colony appears blueish by the nucleic acid stain DAPI, whereas the erythrocytes in the blood agar appear as green dots. E shows the enlarged overlay of the DAPI (blue) and EUB338 (orange) signals from the marked region in the colony. Note the differential activity of the bacteria in the colony and on the colony surface as indicated by the different EUB338 signal intensity. F shows in black and white the region boxed in E (separate DAPI channel). The inset (G) shows in orange the EUB338 signal at this particular position. Note again the different morphologies of the single bacteria ranging from coccoid to pleomorphic.

The **lower middle panel** (**H**–**L**) shows an in vitro grown biofilm of *Rothia dentocariosa*. The bacteria were grown for 48 h on a Polyurethane (PU) chip in a shaking culture with TSB medium. The chip with attached biofilm was embedded in methacrylate and analyzed by FISH. H shows an overview of the biofilm with the DAPI (blue) and the EUB338 (orange) signals overlaid. The region boxed is enlarged (J) again with an overlay of both channels. The indicated region (K) is shown with the EUB338 channel (orange) for this particular position in the biofilm. Even more pronounced note here the pleomorphic morphology of the bacteria in the young biofilm (L).

The **lower panel** (**M**–**P**) shows an in vitro grown biofilm that was left to mature for 7 days on a PU chip in a shaking culture with TSB medium. The panels show identical channels as described for the 48 h biofilm (see above, lower middle panel). Interestingly, note the more coccoid appearance of the bacteria as compared to the younger biofilm (O, P).

Taken together, we conclude that the morphology of *Rothia dentocariosa* may vary substantially depending on the culture conditions. Therefore, the Gram-stain of *Rothia dentocariosa* may be difficult to interpret in the clinical setting where the growth condition of the bacteria must be unknown. Based on the limited number of experiments performed here, it is tempting to speculate if the rod-shape of the bacteria might be associated with younger cultures, whereas older cultures tend to feature more coccoid forms.

(Thiyagarajan et al., 2013). To address possible biofilms and concerns of ongoing infection, in one case, treatment was extended to additional 12 weeks of oral rifampicin and ciprofloxacin after 10 weeks of intravenous antibiotic therapy (Crowe et al., 2014). In our *Rothia dentocariosa* case, the empiric antibiotic therapy for prosthetic IE was administered intravenously for two weeks and was then switched to oral Penicillin V for a further two weeks. The recommended treatment in the reported cases includes four to six weeks of parenteral penicillin, with or without gentamicin, or monotherapy with ceftriaxone (Willner et al., 2019).

Due to the small number of reported invasive *Rothia* infections and lack of MIC cut-off values, antibiotic therapy is chosen according to in-vitro-susceptibility testing and PK/PD-data of the tested substances. Current treatment guidelines recommend the use of a penicillin-derivative in penicillin-susceptible, Gram-positive IE, combined with an aminoglycoside in cases of *Enterococcus faecalis* or penicillin-resistant streptococci. So far, addition of biofilm-active antibiotics (i.e. rifampicin) has been advised in *S. aureus* associated prosthetic valve only, but not in native valve IE (Habib et al., 2015; Baddour et al., 2015). Rieg et al. recently showed, that combination of a beta-lactam antibiotic with either rifampicin or fosfomycin was not associated with improved survival or reduction of late complications in 129 cases of *S. aureus* native valve IE. Interestingly, mortality was significantly decreased with combination therapy when an implanted foreign body was present in a large cohort of *S. aureus* bloodstream infections with high risk for complications (Rieg et al., 2020). A recent study demonstrated non-inferiority of switching to oral antibiotics after 10 days of antibiotic treatment to continued intravenous administration (Iversen et al., 2019). Since the study was only performed on IE by streptococci, *Enterococcus faecalis*, *S. aureus*, or coagulase-negative staphylococci, it remains unclear whether these observations can also be applied to *Rothia* species.

Various mechanisms are known to be involved in the antibiotic tolerance of biofilm building pathogens. Due to the biofilm-forming properties of *Rothia aeria* and *Rothia dentocariosa*, the use of biofilm-effective antibiotics should be discussed. Here, however, further studies are needed confirming the efficacy of anticipated anti-biofilm antibiotic substances in vivo.

5.1. Limitations

Since the constellation examined is rare, our results are based on samples from two patients only.

5.2. Conclusions

In rare cases, IE can be caused by *Rothia aeria* or *Rothia dentocariosa*. The biofilm potential of *Rothia* sp. must be taken into account. In cases of upper airway infections or odontogenic lesions, the awareness of *Rothia aeria* or *Rothia dentocariosa* as relevant pathogens for IE must be raised.

Transparency declaration

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Authors’ contributions

DG, JK and AM made substantial contributions to the conception and design of the work. DG and JK took the lead in writing the manuscript. Specimen processing, fluorescence in situ hybridization and gene amplification and sequencing were carried out by LK and JK. DG, MCK, FP, MST, HG and VF were involved in the clinical treatment of the presented cases. All authors provided critical feedback and helped shape the research, analysis and manuscript.

References

Aistleitner, K., Jeske, R., Wölfel, R., Wießner, A., Kikhney, J., Moter, A., et al., 2018. Detection of Coxiella burnetii in heart valve sections by fluorescence in situ hybridization. J. Med. Microbiol. 67 (4), 537–542.
Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56 (6), 1919–1925.
Baddour, L.M., Wilson, W.R., Bayer, A.S., Fowler Jr., V.G., Tleyjeh, I.M., Rybak, M.J., et al., 2015. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American Heart Association. Circulation 132 (15), 1435–1486.
Cahill, T.J., Prendergast, B.D., 2016. Infective endocarditis. Lancet 387 (10021), 882–893.
Collarino, R., Vergeylen, U., Emeraud, C., Latournèrie, G., Grall, N., Mammeri, H., et al., 2016. Mitral endocarditis due to Rothia aeria with cerebral haemorrhage and femoral mycotic aneurysms, first French description. New Microbes New Infect. 13, 40–42.
Crowe, A., Ding, N.S., Yong, E., Sheorey, H., Waters, M.J., Daffy, J., 2014. Rothia aeria mitral valve endocarditis complicated by multiple mycotic aneurysms: laboratory identification expedited using MALDI-TOF MS. Infection 42 (2), 419–423.
Falcone, E.l., Zelazny, A.m., Holland, S.m., 2012. Rothia aeria neck abscess in a patient with chronic granulomatous disease: case report and brief review of the literature. J. Clin. Immunol. 32 (6), 1400–1403.
Fournier, P.E., Thuny, F., Richet, H., Lepidi, H., Casalta, J.P., Arzouni, J.P., et al., 2010. Comprehensive diagnostic strategy for blood culture–negative endocarditis: a prospective study of 819 new cases. Clin. Infect. Dis. 51 (2), 131–140.
Geissdörfer, W., Moos, V., Moter, A., Loddenkemper, C., Jansen, A., Tandler, R., et al., 2012. High frequency of Tropheryma whipplei in culture-negative endocarditis. J. Clin. Microbiol. 50 (2), 216–222.
Georg, L.K., Brown, J.M., 1967. Rothia, gen. nov. an aerobic genus of the family Actinomycetaceae. Int. J. Syst. Evol. Microbiol. 17 (1), 79–88.
Gescher, D.M., Kovacevic, D., Schmiedel, D., Siemoneit, S., Mallmann, C., Halle, E., et al., 2008a. Fluorescence in situ hybridisation (FISH) accelerates identification of Gram-positive cocci in positive blood cultures. Int. J. Antimicrob. Agents 32, S51–S59.
Gescher, D.M., Mallmann, C., Kovacevic, D., Schmiedel, D., Borges, A.C., Schweickert, B., et al., 2008b. A view on Bartonella quintana endocarditis–confirming the molecular diagnosis by specific fluorescence in situ hybridization. Diagn. Microbiol. Infect. Dis. 60 (1), 99–103.
Habib, G., Lancellotti, P., Antunes, M.J., Bongiorni, M.G., Casalta, J.-P., Del Zotti, F., et al., 2015. 2015 ESC Guidelines for the management of infective endocarditisThe Task Force for the Management of Infective Endocarditis of the European Society of Cardiology (ESC)Endorsed by: European Association for Cardio-Thoracic Surgery (EACTS), the European Association of Nuclear Medicine (EANM). Eur. Heart J. 36 (44), 3075–3128.
Hall-Stoodley, L., Stoodley, P., Kathju, S., Høiby, N., Moser, C., William Costerton, J., et al., 2012. Towards diagnostic guidelines for biofilm-associated infections. FEMS Immunol. Med. Microbiol. 65 (2), 127–145.
Hiraiwa, T., Izumi, M., 2013. Successful management of Rothia aeria endocarditis with renal transplantation patient: a case report. Eur. J. Intern. Med. 24, e204.
Iversen, K., Ihlemann, N., Gill, S.U., Madsen, T., Elming, H., Jensen, K.T., et al., 2019. Partial oral versus intravenous antibiotic treatment of endocarditis. N. Engl. J. Med. 380 (5), 415–424.
Kim, U.-J., Won, E.J., Kim, J.-E., Jang, M.-O., Kang, S.-J., Jang, M.-C., et al., 2014. Rothia aeria infective endocarditis: a first case in Korea and literature review. Ann. Lab. Med. 34 (4), 317–320.
Kronvall, G., Lanner-Sjoberg, M., von Stedingk, L.V., Hanson, H.S., Pettersson, B., Falsen, E., 1998. Whole cell protein and partial 16S rRNA gene sequence analysis suggest the existence of a second Rothia species. Clin. Microbiol. Infect. 4 (5), 255–263.
Li, Y., Kawamura, Y., Fujiwara, N., Naka, T., Liu, H., Huang, X., et al., 2004. Rothia aeria sp. nov., Rhodococcus baikonurensis sp. nov. and Arthrobacter russicus sp. nov., isolated from air in the Russian space laboratory. Mir. Int. J. Syst. Evol. Microbiol. 54 (3), 827–835.
Mahobia, N., Chaudhary, P., Kamat, Y., 2013. Rothia prosthetic knee joint infection: report and mini-review. New Microbes New Infect. 1 (1), 2–5.
Mallmann, C., Siemoneit, S., Schmiedel, D., Petrich, A., Gescher, D.M., Halle, E., et al., 2010. Fluorescence in situ hybridization to improve the diagnosis of endocarditis: a pilot study. Clin. Microbiol. Infect. 16 (6), 767–773.
Michon, J., Jeulin, D., Lang, J.M., Cattoir, V., 2010. Rothia aeria acute bronchitis: the first reported case. Infection 38 (4), 335–337.
Moter, A., Leist, G., Rudolph, R., Schrank, K., Choi, B.K., Wagner, M., et al., 1998. Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiology 144 (Pt 9), 2459–2467.
Nambu, T., Tsuzukibashi, O., Uchibori, S., Yamane, K., Yamanaka, T., Maruyama, H., et al., 2016. Complete genome sequence of Rothia aeria type strain JCM 11412, isolated from air in the Russian Space Laboratory Mir. Genome Announc. 4 (6).
Nicodemo, A.c., Gonçalves, L.g., Odongo, F.c.a., Martino, M.d.v., Sampaio, J.l.m., 2014. Rothia aeria endocarditis in a patient with a bicuspid aortic valve: case report. Braz. J. Infect. Dis. 18 (5), 561–564.
Pape, J., 1979. Infective endocarditis caused by Rothia dentocariosa. Ann. Intern. Med. 91 (5), 746.

Ramanan, P., Barreto, J.N., Osmon, D.R., Tosh, P.K., 2014. Rothia bacteremia: a 10-year experience at Mayo Clinic, Rochester, Minnesota. J. Clin. Microbiol. 52 (9), 3184–3189.

Rieg, S., Ernst, A., Peyerl-Hoffmann, G., Joost, I., Camp, J., Hellmich, M., et al., 2020. Combination therapy with rifampicin or fosfomycin in patients with Staphylococcus aureus bloodstream infection at high risk for complications or relapse: results of a large prospective observational cohort. J. Antimicrob. Chemother. 75 (8), 2282–2290. https://doi.org/10.1093/jac/dkaa144.

Stewart, P.S., Zhang, T., Xu, R., Pitts, B., Walters, M.C., Roe, F., et al., 2016. Reaction-diffusion theory explains hypoxia and heterogeneous growth within microbial biofilms associated with chronic infections. NPJ Biofilms Microbiomes 2, 16012.

Sutrave, S., Kikhney, J., Schmidt, J., Petrich, A., Wiessner, A., Kursawe, L., et al., 2019. Effect of daptomycin and vancomycin on Staphylococcus epidermidis biofilms: an in vitro assessment using fluorescence in situ hybridization. PLoS One 14 (8), e0221786.

Taira, Y., Aoki, Y., 2019. Tube-ovarian abscess caused by. BMJ Case Rep. 12 (8).

Tarumoto, N., Sujino, K., Yamaguchi, T., Umeyama, T., Ohno, H., Miyazaki, Y., et al., 2012. A first report of Rothia aeria endocarditis complicated by cerebral hemorrhage. Intern. Med. 51 (23), 3295–3299.

Thiyagarajan, A., Balendra, A., Hillier, D., Hatcher, J., 2013. The first report of survival post Rothia aeria endocarditis. BMJ Case Rep. 2013.

Tsuzukibashi, O., Uchibori, S., Kobayashi, T., Umezawa, K., Mashimo, C., Nambu, T., et al., 2017. Isolation and identification methods of Rothia species in oral cavities. J. Microbiol. Methods 134, 21–26.

Verrall, A.J., Robinson, P.C., Tan, C.E., Mackie, W.G., Blackmore, T.K., 2010. Rothia aeria as a cause of sepsis in a native joint. J. Clin. Microbiol. 48 (7), 2648–2650.

Willner, S., Imam, Z., Hader, I., 2019. Endocarditis in an unsuspecting host: a case report and literature review. Case Rep. Cardiol. 2019, 7464251.

Yaban, B., Kikhney, J., Musci, M., Petrich, A., Schmidt, J., Hajduczenia, M., et al., 2020. Aerococcus urinae - A potent biofilm builder in endocarditis. PLoS One 15 (4), e0231827.