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In-vitro Study and *In-vivo* Application of Rileva IVD System for the Isolation and Cultivation of Bacterial Strains from Biofilm

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Summary

Introduction: Diagnosis of infection is often complicated by the inability to isolate the pathogens, due to their ability to aggregate into complex structures known as biofilms. This article collects in-vitro and in-vivo evidence of the efficacy of an in-vitro diagnostic (IVD) (RILEVA, Joint Srl, Venice) for the harvesting of explanted prostheses and peri-implant tissues, specifically designed for biofilm breakdown.

Methods: To prove the in-vitro efficacy of the RILEVA IVD, the biofilm of the bacteria E. coli, P.aeruginosa [Gram+] and S.epidermidis [Gram-] was grown for 14 days both on polyethylene (PE) and titanium (Ti) discs. The discs were then placed in the polyvinyl chloride (PVC) bag of RILEVA with a solution of Dithiothreitol (DTT). The solution was processed and centrifuged; the pellet was cultured for up to 10 days on Petri dishes. Data from the clinical use of IVD in 28 cases were analyzed to confirm the in-vitro evidence.

Results: Colonies of all three tested strains of bacteria grew abundantly on the Petri dishes with high colony density in all six replicates, indicative of RILEVA's effectiveness in disrupting the biofilm and identifying the pathogen. Results from the clinical use of the IVD demonstrated the efficacy, confirming in 96.4% of the cases the pre/intraoperative diagnosis.

Conclusion: The RILEVA system, an IVD device that uses DTT to dissolve the biofilm matrix and release sessile bacteria present in the prosthesis, effectively identified the causative agent of prosthetic infection, both in-vitro and when used in a clinical setting.

Keywords: RILEVA; IVD; Prosthetic infection; Biofilm; Bacterial species

Abbreviations: IVD: In-Vitro Diagnostic; Ti: Titanium; PJI: Prosthetic Joint Infection; PVC: Polyvinyl Chloride; PE: Polyethylene; BHI: Brain Heart Infusion; CV: Crystal Violet

Introduction

One of the most common complications of orthopedic implants is prosthetic joint infection (PJI). Depending on the anatomic location, PJI occurs in 1% to 2% of primary joint arthroplasties [1-3], and is associated with individual suffering, increased mortality, and a high economic burden on healthcare systems. In addition, PJI is an escalating problem as the world population ages and the need for joint replacement raises due to increased life expectancy and mobility at older ages [4,5]. Diagnosis of PJI is difficult [6,7], and although many different clinical parameters may indicate the presence of infection, only intraoperative identification of the infecting microorganism provides the highest degree of diagnostic certainty and is therefore necessary to make informed decisions about surgical and medical treatment strategies in the event of infection [7]. Intraoperative histopathological examination of the periprosthetic tissue is used to decide if revision arthroplasty vs resection arthroplasty should be performed when the preoperative evaluation has failed to confirm the prosthetic infection. However,

the results can be dependent on appropriate sampling of the tissue harboring the infection and the expertise of the pathologist, as no guidelines have been developed to standardize cultures to identify pathogens causing the infection [7]. Periprosthetic tissue sampling and aspiration of synovial fluid for subsequent culture are the currently accepted methods for diagnosing PJI [8] but both diagnosis and treatment are difficult and often unsuccessful: the presence of biofilms covering and protecting bacteria on the surface of implanted biomaterials leads to the failure to detect the pathogen and prevents the selection of the appropriate antimicrobial treatment, resulting in recurrence of infection [9]. Therefore, removing the biofilm is of utmost importance to facilitate a clear diagnosis of the organism responsible for the infection [10]. Among the various biofilm removal techniques developed to improve the diagnosis of PJI, sonication of removed prostheses has been introduced in the last decade and is widely used today [11]. Most published reports have shown a higher sensitivity of sonication over conventional periprosthetic tissue cultures for the microbiological diagnosis of PJI [11,12]. However, sonication also has its limitations, such as the low sensitivity in early infections, the lack of a universal sonication protocol, the risk of bacterial contamination from the water in the sonication device, and the requirement for expensive sonication devices [13-16].

In recent years, chemical treatment of removed implants and periprosthetic tissue with dithiothreitol (DTT) has been introduced as an effective alternative to sonication [17-19]. DTT is a sulfhydryl compound that acts as a reducing agent to cleave protein disulfide bonds between cysteine groups, allowing removal of bacterial biofilm from prosthetic implants [20]. DTT has been reported to reduce Staphylococcus aureus biofilm formation [21], and several publications have described the advantages of DTT due to its ease of use, low cost and ability to treat both periprosthetic tissue samples and implants [17,20,22]. RILEVA is a new in-vitro diagnostic (IVD) device for the sterile collection and processing of explanted prostheses and/or solid peri-implant tissues that are infected or suspected of being infected. RILEVA is supplied in the form of a bag that allows sterile handling of prostheses and explanted tissues in the operating room and their transport to the microbiology laboratory where they are processed. The risk of contamination is very low as sample manipulation is minimal, reducing the risk of false positive results. The action of RILEVA is based on the ability of the DTT solution in which the explanted prosthesis is bathed to dissolve the biofilm matrix and release the bacteria present in sessile form. In this study, we investigated the efficacy of RILEVA IVD to obtain bacterial strains from biofilms grown in prosthetic-like material in-vitro. In addition, we investigated the performance of RILEVA in a clinical setting, in the collection of intraoperative samples to identify infecting bacteria from surgically explanted prostheses and compared the results with those obtained with conventional microbiological analysis of wound exudate/biological fluid performed pre- and intraoperatively.

Materials and Methods

Biofilm growth

Escherichia coli (ATCC® 39327), Pseudomonas aeruginosa (ATCC® 35695) and Staphylococcus epidermidis (ATCC® 12228) were grown in their specific media, EC broth (Sigma Aldrich), Cetrimide broth (Sigma Aldrich) and Staphylococcus broth (Sigma Aldrich), respectively, at 30 °C in a shaking incubator (300 rpm). After 24 hours, strains (1 mL) were transferred individually to liquid Brain Heart Infusion (BHI) and incubated in a shaking incubator at 30 °C for five days. The strains thus cultured were inoculated individually (500 µl) in triplicate onto custom-designed sterile polyethylene and titanium discs and incubated at 30°C under static conditions. After six days, the discs were again inoculated with the same bacterial broth and incubated at 30 °C for one day. Subsequently, the discs were placed in a shaking incubator (150 rpm) at 30 °C for another seven days to stimulate biofilm growth.

Evaluation of biofilm growth

Biofilm formation was evaluated using a colorimetric method [23]. The biofilm-containing discs were rinsed four times with 500 µl sterile water to remove non-adhering cells. The biofilm was fixed with 500 µl of 96% ethanol and air dried for 15 minutes. 500 µl of a 0.2% (w/v) solution of crystal violet (CV) was poured onto the disc surface which was then stained for 20 minutes. The excess solution was discarded to visually observe the stained biofilm.

RILEVA system for biofilm removal

The IVD RILEVA system includes

i. n. 1 sterile polyvinyl chloride (PVC) bag with an inlet and outlet port with cap

ii. n. 1 polyammide sterile clamp for the hermetic sealing of the bag

iii. n. 4 sterile 60 mL syringes

iv. n. 2 sterile10 mL syringes

v. n. 4 sterile 19 G needles

vi. n. 2 sterile 50 mL Falcon with screw cap

vii. n. 1 apyrogenic 100 mL water bottle

viii. n. 1 1,4-ditiotreitol (DTT) 100 mg bottle

ix. n. 1 bag for biological sample transportation

To test the effect of DTT and RILEVA IVD on biofilm removal, biofilm-containing discs were placed in the PVC bag and sealed with the clamp as indicated by the manufacturer. One bag contained both the polyethylene and titanium discs previously inoculated with one of the selected strains. Then, 10 mL of apyrogenic water was withdrawn with a 10-mL syringe and mixed with DTT, which was then diluted with the remaining water (90 mL). The solution (100 mL) was injected into the disc bag through

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the inlet port, and the bag was placed on an oscillating plate (40 rpm) for 10 minutes. Then the solution was withdrawn through the outlet port and centrifuged at 4100 rpm for 10 minutes in the two screw-capped Falcon tubes. The supernatant was removed, and the resulting pellet was applied to the specific agar media in Petri dishes and incubated at 30 °C under static conditions for up to 10 days. A control experiment was performed using the same procedure with a 0.9% physiological NaCl solution instead of DTT.

Evaluation of RILEVA in the clinical setting

Implants from 28 patients with overt or suspected implant-related infections (plates/screws 28.6%, knee 17.8%, hip 14.3%, shoulder 14.3%, elbow 14.3%, other implants 10.7%) were surgically explanted. Intraoperative specimen collection using the RILEVA IVD includes several steps:

a. opening the pouch and clamp using sterile technique and placing them on a cart away from the surgical instruments.

b. placing all explanted material directly into the pouch using clean instruments.

c. sealing the pouch with the clamp after explantation by placing it as close to the material as possible to reduce the internal volume of the pouch.

d. removing the pouch from the sterile field and attaching the patient label ID.

e. placing the pouch in the transport bag for biological samples and sending it to the microbiology laboratory where the specimens are subjected to microbiological testing.

The surgical procedure was performed by the Department of Orthopedics and Traumatology in collaboration with the Department of Microbiology of the Ospedale dell'Angelo di Venezia-Mestre for the processing of the specimens with the RILEVA system. Preoperative and intraoperative microbiological analysis of wound exudate/biological fluid was performed in all patients using conventional culture methods. Microbiological analysis of the removed prostheses was also performed in all patients using the RILEVA system, closely following the manufacturer's instructions.

Results

E.coli, P. aeruginosa, and S. epidermidis were grown on polyethylene or titanium discs. A colorimetric assay using crystal violet (CV) was performed to evaluate the growth and stability of the bacterial biofilm. Figure 1A shows the E.coli biofilm before and Fig. 1B after the colorimetric assay. The formation and growth of the biofilm on the discs is evident by the presence of a dark purple coloration on the disc surface (Figure1B, center and right Petri dishes), which is not present in the absence of the inoculated bacteria (Figure 1B, left Petri dishes). This result confirms that biofilm formation occurs under our experimental conditions.

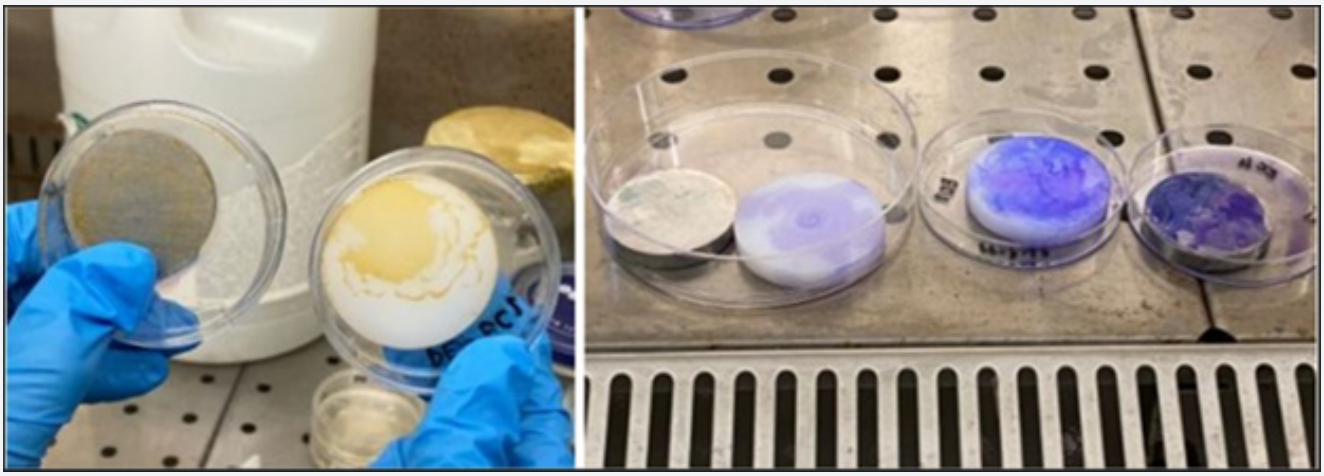

Figure 1: Biofilm after 14 days prior (left) and after (right) the crystal violet experiment. On the right, the differences between discs with and without (blank) biofilm are visible.

Next, we tested whether processing the discs after biofilm growth with RILEVA IVD in strict accordance with the manufacturer's instructions would allow isolation of the bacterial strains. Discs inoculated with bacteria were exposed to the DTT-containing solution and the solution was then centrifuged. The resulting pellet was then applied to a Petri dish containing an appropriate growth medium, and after culturing, the dishes were examined for the presence of bacteria. Figure 2 shows that

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colonies of all three strains of bacteria tested grew on the Petri dishes, and the high colony density in all six replicates indicates a good recovery rate. The growth of all strains indicated the successful removal of biofilm from the dishes, suggesting that the use of RILEVA IVD with DTT enables the isolation and cultivation of biofilm bacteria from prosthetic materials such as polyethylene and titanium.

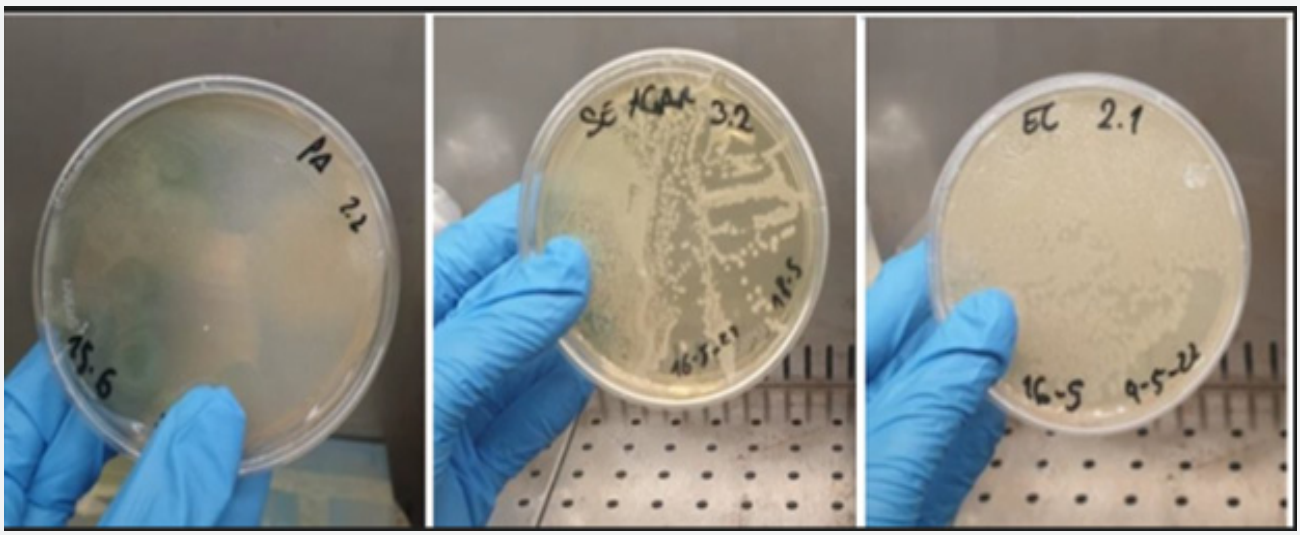

Figure 2: Colony growth of P. aeruginosa (left), S. epidermidis (centre) and E. coli (right).

To further investigate the role of DTT in biofilm removal, a control experiment was performed with the RILEVA IVD system, replacing DTT with a physiological solution. Under these conditions, E.coli was detected in only 1 of 3 replicates. The presence of only a few E.coli colonies also suggests that the use of the physiological solution instead of DTT significantly reduces the ability to isolate this strain. S. epidermidis also showed lower growth under control conditions, although colonies were clearly detectable. However, P. aeruginosa was found to grow in all replicates using the physiological solution, like the DTT solution. This strain-dependent growth variability suggests that the use of the physiological solution may not ensure recovery of all bacteria strains and reproducibility, increasing the likelihood of false negative results. Taken together, the above results show that the performance of the DTT-based method for biofilm removal was superior to that of a physiological solution in isolating Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus epidermidis cultures.

Clinical setting

The ability of RILEVA to isolate biofilm bacteria in-vitro was also confirmed in a clinical context by evaluating its diagnostic efficacy in isolating and identifying bacteria from explanted prostheses and comparing it to conventional microbiological analysis of wound exudate/biological fluid performed pre- and intraoperatively. The results obtained on 28 patients, 13 women and 15 males, with a mean age of 65.8±11.1 years, show that 27 cases (n=27/28; 96.4%) were consistent with pre/intraoperative diagnosis. In particular, in 22 cases (n=22/28; 78.6%) the RILEVA system allowed to detect the pathogen involved in the infection: in 17 cases (n=17/22; 77.3.0%) the pathogen(s) was the same detected in previous analysis, in four cases (n=4/22; 18.2%) RILEVA enabled isolation of the infectious microorganism in the presence of negative or absent pre/postoperative diagnosis, and in one case the bacteria detected through the RILEVA system was different from the one observed previously. The five negative cases (n=5/28; 17.9%) obtained with the RILEVA system agreed with clinical data and confirmed the absence of infection. In only one case (n=1/28; 3.6%) the negative result obtained with RILEVA was not consistent with conventional pre/intraoperative testing (Figure 3).

Discussion

In this study, we show that RILEVA, an in-vitro diagnostic (IVD) device that uses DTT to dissolve the biofilm matrix and release sessile bacteria present in the prosthesis, effectively identifies the causative agent of prosthetic infections, both in-vitro and when used in a clinical setting and is more accurate than conventional culture methods. The pathogenesis of prosthetic infection has been shown to depend on the formation of a biofilm. Biofilms are complex communities of microorganisms embedded in an extracellular matrix that form on the surface of prosthetic

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implants. Pathogens adhere to orthopedic devices, multiply, and produce exopolysaccharides that coalesce over time to form a biofilm. The biofilm microenvironment serves as a barrier against the host's endogenous defense system or external agents such as antibiotics [24,25]. In addition, pathogens that colonize biofilm have low metabolic rates that prevent accurate identification in culture. S. epidermidis, Pseudomonas aeruginosa, and S. aureus are the most common biofilm-forming bacteria found in medical devices [26]. Biofilm formation not only plays a role in the pathogenesis and treatment of infection, but also has implications for the diagnosis. Particularly in delayed and late onset of prosthetic infection, the causative pathogens are concentrated on the surface of the prosthesis, which reduces the sensitivity of periprosthetic tissue and fluid cultures. A retrospective evaluation examining 35 patients showed that preoperative joint aspiration is likely to miss some bacterial species [27], and conventional methods of synovial fluid and tissue sample culture have a high rate of false-negative and false-positive results [28].

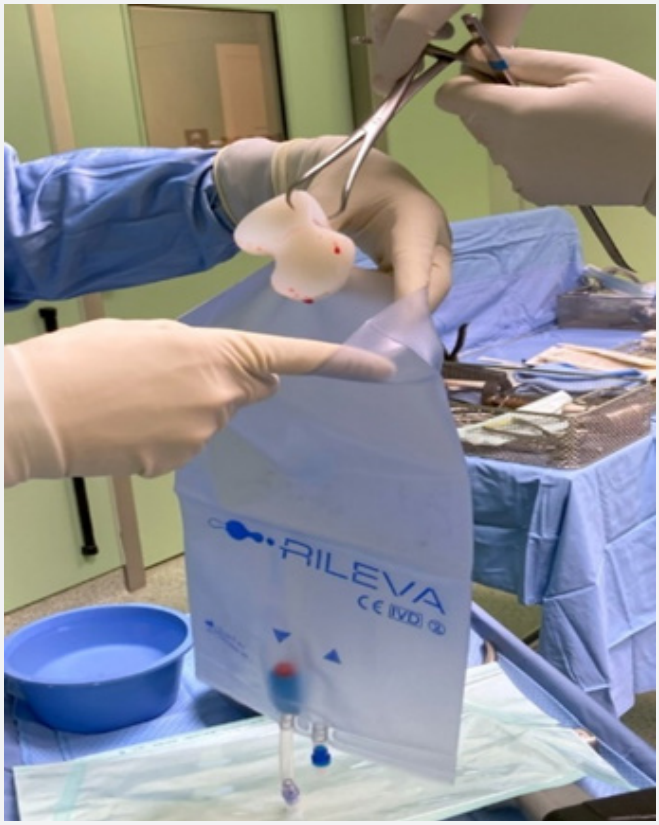

Figure 3: Intraoperative image of RILEVA IVD used after orthopedic prosthesis explantation for subsequent biofilm analysis.

One strategy to overcome this limitation is to directly sample the surface of the prosthesis taking advantage of techniques such as sonication or DTT treatment to dislodge the microorganisms adhering to the prosthetic joint surface. A study investigating the applicability of DTT for the treatment of periprosthetic and osteoarticular tissues for the diagnosis of bone and joint infections found that treatment with DTT had higher sensitivity and specificity compared with normal saline, suggesting its usefulness in the diagnosis of bone and joint infections [17]. In another study comparing DTT treatment with sonication, it was found that the two methods produced similar results in terms of bacterial yield, with DTT having the same specificity and better sensitivity than sonication [22]. However, in a randomized trial designed to determine whether DTT and sonication are more sensitive and/or specific than standard culture methods in diagnosing PJI, no differences in sensitivity were observed between DTT and sonication, but both were found to be more sensitive than standard culture methods [29]. In a prospective study examining explanted implants from 73 cases of revision arthroplasty, DTT treatment was also shown to be effective in diagnosing PJI and had higher sensitivity and comparable specificity to sonication [19]. Both sonication and DTT have been shown to be more efficient than conventional bacterial culture. However, sonication requires a specialized and expensive device, whereas DTT is easy to use, very inexpensive, poses fewer environmental risks, and can be applied to both implants and periprosthetic tissue [20]. A disadvantage of DTT is its potential toxicity to bacterial cells, which in principle could bias the results of DTT-based biofilm removal and lead

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to false-negative results [20]. However, our findings contradict this notion and suggest that DTT is more effective than saline in extracting bacteria that can be cultured from the biofilm of explanted prostheses.

Conclusion

In summary, our study shows that the RILEVA device using DTT is very reliable for the isolation of bacterial strains from biofilm adhering to titanium or polyethylene discs, whereas the isolation of bacteria using conventional methods is less reproducible. In addition, RILEVA also proved easy to use and reliable in the clinic. When tested on explanted implants, it consistently allowed isolation of various contaminating bacterial species, and in most cases the results correlated positively with those obtained with conventional pre/intraoperative microbiological testing and/or clinical evaluation of the patient. Importantly, in some cases, only RILEVA provided an accurate microbiological diagnosis of PJI. Thus, culturing DTT extracts from removed prostheses could play an important role in the workflow for the diagnosis of PJI, but larger data sets from controlled, multicenter studies are needed to validate these findings.

Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Forghieri Giulia, Menegazzo Federica and Signoretto Michela conceived, planned and carried out the in-vitro experiment. Ussia Giuseppe and Miti Andrea provided the explanted samples to the Microbiology department. Solinas Maria and Scarparo Claudio examined the explanted samples. All the authors discussed the results and contributed to the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement

Not applicable.

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