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Summary: The diagnosis of infectious diseases by metagenomic shotgun sequencing is limited by background DNA. We show here, in a case of blood culture-negative endocarditis, that microdissection can help overcome this obstacle when microbes are visualid in tissues.

ABSTRACT

Background

Etiological diagnosis is one of the keys to therapeutic adaptation and improved prognosis, particularly for infections such as endocarditis. In blood-culture negative endocarditis (BCNE), 22% of cases remain undiagnosed despite an updated comprehensive syndromic approach prompting us to develop a new diagnostic approach.

Methods

Eleven valves of 10 BCNE patients were analyzed using a method combining human RNA bait-depletion followed by phi29 DNA polymerase-based multiple displacement amplification and shotgun DNA sequencing. An additional case, in which the microbe was serendipitously visualized by immunofluorescence, was analyzed by the same method, but after laser capture microdissection (LCM).

Results

Background DNA prevents any diagnosis in the cases analyzed without microdissection because majority sequences were contaminants. *Moraxella* sequences were dramatically enriched in the stained microdissected region of the additional case. A consensus genome sequence of 2.4Mbp covering more than 94% of the *Moraxella osloensis* KSH reference genome was reconstructed with a 234X average coverage. Several antibiotic resistance genes were observed. Etiological diagnosis was confirmed by western blot and specific PCR with sequencing on a different valve sample.

Conclusion

Microdissection could be a key to the metagenomic diagnosis of infectious diseases when a microbe is visualized but remains unidentified despite an updated optimal approach. *M. osloensis* should be tested in blood-culture negative endocarditis.

Keywords: Blood culture negative endocarditis, laser capture microdissection, Human RNA-bait depletion, *Moraxella osloensis*

INTRODUCTION

Etiological diagnosis of infectious diseases is one of the keys to therapeutic adaptation and improved prognosis, particularly for infections such as endocarditis. Endocarditis without etiological diagnosis represent 22% of the cases [1]. We have recently developed a multimodal syndromic strategy that includes blood and valves analysis when available using serology, molecular diagnosis by broad spectrum PCR, specific real-time PCR and anatomopathology by immunohistochemistry (IHC) [2,3]. In our previous study, we showed that molecular diagnosis and specific real-time quantitative polymerase chain reaction (qPCR) improved diagnostic efficiency by 24%, mainly by detecting enterococci and streptococci that had not been detected by other diagnostic tools [1], including by broad range 16S rRNA amplification and sequencing. Thus, although these bacteria are easily cultivable, only molecular biology allows their identification in a certain number of cases, notably when the patient has received prior antimicrobial therapy.

Other authors have developed a syndromic strategy using Fluorescent In Situ Hybridization (FISH) on the excised cardiac valve with a universal probe (EUB338) and 10 specific probes with very promising results [4]. However, in at least 1 case, the universal probe showed a microbe that could not be identified by the 10 specific probes. In such a situation, a non-targeted approach could be helpful.

As an expert center in the diagnosis of blood culture negative endocarditis (BCNE) [5], we developed microdissection in the context of an ongoing unsuccessful work using metagenomics shotgun sequencing to analyze 11 valve tissues from 10 patients with blood

culture-negative endocarditis. In this preliminary work, the background DNA [6,7] prevented any robust diagnostic. In this context, we had the opportunity to analyze the microdissected valve of a single patient for whom the anti-*Coxiella burnetii* IHC was positive [3], while our comprehensive syndromic approach was negative, including culture, serology, broad range PCR targeting the 16S rRNA gene and multiple specific real-time qPCR targeting usual endocarditis pathogens, including *C. burnetii*.

We used laser micro-dissection [8] on IHC positive and negative sections that were further submitted to DNA shotgun next-generation-sequencing (NGS) before and after in-solution capture of human nucleic acids using biotinylated RNA-baits. Diagnosis was confirmed using western blot and specific PCR with sequencing on the same excised valve and on another valvular tissue excised during the same procedure.

MATERIAL AND METHODS

Clinical samples

All clinical samples were obtained from the diagnostic laboratories of our center (Hôpital de la Timone, Marseille, France – 11 valves from 10 patients with blood culture negative endocarditis) and from the Bichat hospital (Paris, France – additional case analyzed with microdissection) and analyzed at the Institut Hospitalo-Universitaire Méditerranée Infection (Marseille, France). The local IFR 48 ethics committee approved the consent and study protocol. The authors certified that this study was not in opposition to the declaration of Helsinki and in accordance with the French laws, respectively. The data were analyzed anonymously. The patient's written consent has been obtained.

Human RNA bait-depletion and sequencing

To optimize our ability to identify a putative microbe in valves from blood-culture negative endocarditis patients, we developed a method to deplete human DNA in the sample. This method included a hybridization reaction with a biotinylated human RNA-bait library and capture with DynabeadsTM MyOneTM Streptavidin C1 beads (Thermo Fisher Scientific, Waltham, MA, USA) using a magnetic particle collector as previously described [9]. Full details of Human RNA bait-depletion are provided in the supplementary data. Sequencing was performed. The microbial DNA amount in the positive tube (area with specific immunostaining cut by laser capture microdissection) was expected and observed to be very low. We therefore used a nonspecific DNA amplification method before sequencing. Nucleic acids recovered from the human-depleted fractions were amplified with GenomiPhi (GE Healthcare, Chicago, IL, USA) in duplicate to generate sufficient material for Illumina library preparations. DNA was sequenced on a MiSeq platform using a paired-end strategy according to a Nextera XT library kit in a 2 × 250 bp format (Illumina Inc., San Diego, CA, USA). While developing this human RNA-bait depletion method, we were confronted with an enigmatic case with positive immunohistochemistry for *C. burnetii* (Figure 1) but negative in PCR and serology. Consequently, this single enigmatic case was analyzed by several axenic and cell culture conditions, immunohistochemistry (IHC), fluorescent in situ hybridization (FISH), microdissection-human RNA bait depletion-sequencing, specific PCR and Western blot. Accordingly, the following methods have been performed only for this additional case.

Microbial culture

All samples (heparinized blood, mechanical mitral valve, aortic valve ring, mitral valve vegetation) were inoculated for conventional axenic agar culture and cell culture as detailed in the supplementary data.

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH)

Immunohistochemical detection using a *C. burnetii* monoclonal antibody coupled with an immunoperoxidase was performed as previously described [3]. FISH experiments were conducted as previously described on 3-µm-thick formalin-fixed paraffin-embedded tissue sections [10]. To confirm the specificity of the immunostaining observed, confocal spectral imaging was performed with a Zeiss LSM 780 (Zeiss, Oberkochen, Germany) as previously reported [11, 12] and detailed in the supplementary data.

Laser capture microdissection and sequencing

Laser capture microdissection was performed using an Arcturus® XT system (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (full details are provided in supplementary data). The capture areas were directly collected into a sterile 1.5 ml microcentrifuge tubes containing 180 µl of Lysis Buffer T1 (Macherey-Nagel, Duren, Germany) and were then processed for extraction.

Confirmation techniques

To confirm the infection, we used 1) Western blot that would evidence a specific immunological respond of the patient to the suspected microbe identified by our method and 2) PCR targeting repeated elements of the genome obtained by our method followed by sequencing. Full details of confirmation techniques are provided in the supplementary data.

RESULTS

In-solution capture of human nucleic acids using biotinylated RNA-baits

We first developed a metagenomic approach with DNA shotgun next-generation-sequencing (NGS) before and after in-solution capture of human nucleic acids using biotinylated RNA-

baits on 11 valve samples from 10 patients with blood culture negative endocarditis. The hypothesis was that the pathogen responsible for endocarditis would be represented by the maximum of non-human sequences in the sample. Despite using human DNA depletion method, we first observed that only a small proportion of quality-filtered reads were non-human (mean 9.3%) and only 4.0% of non-human reads were annotated (Supplementary Table 1). Based on non-human annotated reads, the most represented species were most likely to be contaminants.

We therefore sought to clarify the criteria that suggest that a species detected with an acceptable abundance (>30 reads in at least 1 sample) was a contaminant and not the pathogen responsible for the valve lesion and infection. The first criterion we identified was the presence of this species in abundance (more than 10 reads) in the negative control. This first criterion identified 14 phylotypes, including *Cutibacterium acnes, Malassezia globosa* and *M. pachydermatis*, *Homo sapiens*, 7 *Sphingomonas, Brevundimonas* sp. DS20, *Exiguobacterium* sp. S17 and *Treponema denticola*. The second criterion was the fact that the species was found in more than 50% of cases (more than 5 valves). 14 taxa met this criterion: *Staphylococcus epidermidis, Enhydrobacter aerosaccus*, 3 phages of *Pseudomonas*, 2 *Prevotella*, 2 *Streptococcus* (including *Streptococcus mitis* which is an endocarditis agent), *Paenibacillus sophorae, Corynebacterium casei, Lawsonellia clevelandensis* and *Porphyromonas gingivalis*. Four other species (*A. baumanii, Staphylococcus hominis* and *capitis, Malassezia restricta*) were considered contaminants because they were found in high abundance in several experiments, including RNA experiments (data not shown).

After excluding all possible contaminants defined above, no species has been reliably identified in the heart valves (Table 1). Oral bacteria were identified (*Porphyromonas endodontalis, Prevotella denticola*). *Granulicatella elegans,* previously described as an agent of blood culture negative endocarditis, diagnosed only by molecular technique on cardiac

valve [13], was identified in 1 case. In one case, 4 species sharing a common taxonomy (Eukaryota;Opisthokonta;Fungi;Dikarya;Basidiomycota) were identified, suggesting a fungal endocarditis. *Lactobacillus fermentum* was identified in 1 case, this bacterium has been rarely associated with endocarditis in the literature [14]. *Treponema* species were also found (*Treponema maltophilum*). In this context, we had the opportunity to apply this method after microdissection of a valve where the immunohistochemistry was positive for *Coxiella burnetii*, but the serology and PCR were negative.

Genome reconstruction of a new strain of *Moraxella osloensis*

Positive immunostaining using rabbit anti-*C. burnetii* antibodies obtained by immunohistochemistry (Figure 1) was confirmed by immunofluorescence with confocal spectral microscopy (Supplementary Figure 1). After laser capture microdissection (detailed methods and results are provided in the supplementary data – Supplementary Figure 2), capture and nonspecific DNA amplification, the proportion of non-human reads that were taxonomically assigned in the T+ increased from 1.5% (before capture) to 72.3% (after capture - Supplementary Table 2). A possible enrichment in a *Moraxella* species was suspected based on an increase in *Moraxella*-associated reads in the T+ (Supplementary Figure 3-5). In the T+, *Moraxella caprae* was the majority species while in the T-, contaminants, as defined above, were majority (*C. acnes, M. globosa*). In the T+, 25.7% (1678/6521) reads were associated with *Moraxella* species versus 6.1% (3501/56863) in the T- (unstained). Accordingly, microdissection increases by 4.2 the ratio of reads associated with the microbe stained by immunohistochemistry and immunofluorescence. We then focused on identifying the *Moraxella* strain at the species level detected in the positive sample (T+) after human capture. The percentage of reads mapped on the *M. osloensis* KSH reference genome (NZ_CP024180.2) and plasmids (NZ_CP024181.2 to NZ_CP024184.2) increased

from 0.13% (2,725 reads) to 77.6% (2,705,641 reads) after capture of the human nucleic acids.

A genome consensus sequence of 2.4 Mbp (2,348,787 bp, 187 contigs) with a GC content of 44.2% was obtained with an average coverage of 234X. This consensus sequence was aligned against the *M. osloensis* KSH (GenBank assembly accession: GCA_002752795.2) reference genome using progressive MAUVE and annotated with the RAST server. Alignment with the *M. osloensis* KSH reference genome showed synteny conservation (Supplementary Figure 6 & 7) with gaps corresponding to the 4 copies of the 16-23S rRNA genes that generated conflicts in the mapping process (Supplementary Figure 8). 16S rRNA sequences were retrieved using the very sensitive mapping option implemented within Bowtie 2. A consensus sequence that shares 99 to 100% identity with the 4 copies of 16S rRNA gene of *M. osloensis* KSH has been replaced in a phylogenetic tree (Figure 2). This new strain of *Moraxella osloensis* was named *M. osloensis* strain Marseille. The partial genome of the *M. osloensis* strain Marseille was annotated using the RAST server and displayed 2,167 protein-encoding genes (PEGs) and 64 RNAs. Several genes associated with antibiotic resistance were found including genes resistant to colistin, fluoroquinolones, beta-lactamase, tetracycline and macrolide-lincosamide-streptogramin. These genes included particularly MCR_1/2 protein (colistin – Supplementary Figure 9), ICR-Mo (colistin), tetB (tetracycline), optrA (oxazolidinones), OXA-372 (carbapenem) and OqxAB / AcrA/B (multidrug efflux pump). Full details of genomic analysis for antibiotic resistance genes are provided in the supplementary data (Supplementary Text - Supplementary Table 3).

Confirmation of the etiological diagnostic

In order to confirm the patient's infection by *Moraxella osloensis* at the time of cardiac surgery, we performed western blots with the patient’s serum and several *Moraxella* strains

corresponding to the closely related species; *M. osloensis, M. bovoculi, M. caprae,* but also a *Coxiella burnetii* strain. No bands were observed with *Coxiella burnetii* antigens, thus confirming false positive immunohistochemistry and FISH (Figure 3). On the other hand, at least one band was observed for all *Moraxella* strains and the western blot was the most positive for *M. osloensis*, using serum collected at the earliest date of surgery in which the mitral valve vegetation sample was excised (Figure 3).

To confirm the false positive of immunohistochemistry and immunofluorescence, we tested whether the primary antibody used produced in rabbits by *C. burnetii* infection had a cross-reaction with *M. osloensis*. After culturing *M. osloensis* strain CSURP3830, we were able to detect antibody staining used for anti-*C. burnetii* immunohistochemistry and thus confirmed the cross reaction (Supplementary Figure 10 and Supplementary Video 1).

For a highly sensitive and specific detection of *M. osloensis*, specific primers targeting a region conserved and repeated in the *M. osloensis* CCUG 350 reference genome have been designed. These primers (M_oslo_transpF AAATGCGAGAACGCAGGTTG and M_oslo_transpR CCTTTCGGACTATTGGCGGT) amplify a 101 bp fragment of a gene that has 23 genomic copies and is coding for a transposase. A strong positive signal has been detected in the microdissected and random-amplified positive valve sample (Figure 4). A positive signal has also been observed in the original (unamplified) microdissected positive valve sample. In order to exclude contamination during the microdissection process, another tissue section of the same paraffin bloc, for which nucleic acids were directly extracted, was also tested positive for *M. osloensis* and confirmed by sequencing. Finally, a faint PCR signal confirmed by sequencing the presence of *M. osloensis* in nucleic acids extracted from a mitral vegetation resected during the same chirurgical operation and directly frozen for the diagnostic laboratory (Figure 4). All PCR products have been Sanger sequenced and

confirmed as *M. osloensis*. All these confirmatory results suggest a *Moraxella osloensis* endocarditis previously reported in the literature (Table 2).

DISCUSSION

Here, we show that microdissection combined with human DNA depletion is a key approach to control the background DNA in metagenomics shotgun sequencing for infection diagnosis [6,7]. The localization of microbes in infected tissues is one of the main issues for the use of laser microdissection combined with metagenomics in the diagnosis of infectious diseases. This localization involves visualizing microbes with an optical and/or electron microscope, which is simple when they are visible with standard staining or surrounded by a granuloma, but difficult in the absence of adequate staining, when the type of microbe is unknown, when the microbes are small (non-cultivated microbes have a smaller genome than cultivated microbes and are probably smaller [15]) or when the microbes are intracellular. Non-specific fluorescent markers can be used (DAPI, shape and size helps to differentiate eukaryotic and prokaryotic cells [16]). Immunofluorescence improves the sensitivity of detection of labelled cells compared to immunohistochemistry [17], but this also depends on the antibodies used [18], so the 2 methods seem complementary. Silver ions fluorescence probes further increase the sensitivity up to 30 $pg.mL^{-1}$ [19]. It is interesting to note that in the case presented here, the microscopic aspects differ between the two techniques though they target the same antigens (intracellular punctiform images in IF and whole cells stained by IHC). Fluorescence in situ hybridization (FISH) is also a very promising technique [20] whose effectiveness is improved by peptic nucleic acid probes [10]. FISH allows the use of universal probes (EUB338, with negative control by non-EUB338 probe [10,16]) possibly coupled to specific probes [10, 16]. This technique is effective in detecting pathogens in clinical settings in blood cultures [21, 22], but also in cardiac valve tissues of patients with endocarditis [4]. In a study involving 54 endocarditis patients and using universal and endocarditis-specific probes, FISH detected bacteria in 26 cases including 5 of 13 blood culture negative cases and 11 of 37 valve culture-negative cases [4]. In one case, FISH was positive using universal probes (EUB338),

but the microbe remained unidentified despite 10 specific probes targeting genera and species responsible for endocarditis. In this case, our approach could have been valuable, because direct metagenomic analysis of the cardiac valve tissue would have yielded a majority sequences of contaminants ("sequencing noise" or background DNA [6,7]). When the microbes are visualized, microdissection offers the unbeatable advantage to increase the metagenomic signal / noise ratio. The co-localization of a universal or specific probe and DAPI is an additional argument for viable cells [16]. Confocal multicolor spectral confocal microscopy [11,12] could be decisive to confirm the collocation of two signals (IF/FISH). Correlative Light and Electron Microscopy (CLEM) could also be of great help for microbes whose diameter is less than 500 nm and therefore difficult to visualize in optical microscopy such as Candidate Phyla Radiation or intracellular microbes such as *Coxiella* spp. [10,16]. While these techniques for visualization and labelling of microbes coupled with laser microdissection have been very successful in fundamental laboratories [16,23], it is time for this approach to be used for the benefit of patients with difficult diagnostic infections, including negative blood culture endocarditis.

The cost is difficult to assess but the global cost for human RNA-bait capture experiment is about 50 euros per sample. The global cost for nucleic acid extraction, amplification and sequencing (with an average coverage of 2 million paired reads) is about 250 euros per sample. The total is about 300 euros per sample, but sequencing costs are decreasing. However, this does not include the laser microdissection system and the bioinformatic analyses. The other point to raise is the lack of standardization. As far as our approach is concerned, the time required, technical expertise and cost required do not yet seem compatible with a routine procedure. Future studies will have to optimize the cost-benefit ratio and protocols in order to reduce the operator-dependent part and at best to

automate all or part of the procedure. Nevertheless, this approach offers an unprecedented opportunity for diagnosis when all usual methods have failed.

The innovative approach reported here could be applied to any tissues where microbes are visualized under a microscope using standard staining techniques. It could even bring a new future to autoimmunohistochemistry, a technique developed at our center to visualize microbes infecting a patient using a tissue biopsy, its own serum and a labelled human anti-IgG antibody [24]. Until now, the interest of autoimmunohistochemistry has been limited by the fact that the technology to identify labelled microbes was not available. This technique could also be improved using immunofluorescence, which is much more sensitive than immunohistochemistry. 16S rRNA fluorescent in situ hybridization (16S FISH) is another method that could allow microbes to be located before microdissection [4,20]. Here, we are bringing a new technology that could substantially improve the diagnosis of infectious diseases when other techniques have been defeated but a microbe is visualizable with standard staining, fluorescence in situ hybridization, correlative light electron microscopy (CLEM) or auto-immunostaining. This could be the first step of a revolution in the diagnosis of undocumented infections.

NOTES

Authors' contributions. MM, MG, CD and DR wrote the manuscript; CV and RD took care of the patient; CM retrieve the clinical picture and collected the samples; SE has performed standard diagnostic techniques (serology, PCR); BLS did the cell culture; HL did immunohistochemistry; MM, CM, LC, EP, EP, SM have done immunolabelling and fluorescent in-situ hybridization; MF did the confocal spectral microscopy; MM, LC, PN did the laser capture microdissection; MM, MG, CR, CD conducted the metagenomic, biostatistical analysis and human RNA bait-depletion; BA did the Western-blot; MG did the confirmatory PCRs; MM, JPG and DR coordinated the project; DR designed the study.

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Table 1. Metagenomics results after human RNA-bait depletion without microdissection on 11 excised valves from 10 blood culture negative endocarditis patients

Patient_ID	Number of reads assigned at the species level	Species with more than 30 reads
1	1309	*Treponema maltophilum* 115 reads
		Porphyromonas endodontalis[a] 35 reads
2[b]	4196	*Granulicatella elegans* 139 reads[c]
		Corynebacterium kroppenstedtii 81 reads
		Prevotella denticola[a] 48 reads
		Corynebacterium tuberculostearicum 42 reads
3	1432	No species with more than 30 reads
4	1093	No species with more than 30 reads
5	70376	*Tilletiaria anomala*[d] 145 reads
		Anthracocystis flocculosa[d] 135 reads
		Jaapia argillacea[d] 81 reads
		Gelatoporia subvermispora[d] 32 reads
6	737	*Corynebacterium propinquum* 44 reads
7	2334	*Paracoccus yeei* 64 reads
8	1552	No species with more than 30 reads
9	464	*Lactobacillus fermentum*[e] 42 reads
10	414	No species with more than 30 reads

[a]Bacteria associated with oral microbiota, [b]The second valve sample from this patient yielded no species with more than 30 reads after excluding probable contaminants, [c]We previously described this microbe as an agent of blood culture negative endocarditis and diagnosed only by molecular technique on cardiac valve [13]. [d]All these 4 species shared a common taxonomy (Eukaryota;Opisthokonta;Fungi;Dikarya;Basidiomycota). [d]We found 1 case of *L. fermentum* endocarditis in the literature [14].

Table 2. Documented cases of infective endocarditis due to *M. osloensis* since 1967

Country	Patient	Type of endocarditis	Concurrent condition/Clinical history	Detection	Treatment	Evolution	Reference
USA, 1982	66-years old male	Prosthetic aortic valve	renal failure	Blood culture	Penicillin, oxacillin, tobramycin	Died	[25]
France, 2015	75-years old male	Native aortic valve	B-cell chronic lymphocytic leukaemia	Histopathology,culture of native valve, MALDI-TOF, 16S sequencing	amoxicillin/gentamicin	Cured	[26]
France, 2015	51-years old male	Aortic abcess, prosthetic mitral valve	IE on prothetic mitral valve, Hodgkin's lymphoma	Culture of Prosthetic valve, MALDI-TOF, 16S sequencing	Cefotaxime	Cured	[26]

			and a kidney transplant				
Brazil, 2018	41-years old male	Native mitral valve	None	Blood culture	vancomycin, gentamicin, ampicillin, ceftriaxone	Cured	[27]
France	38-years old female	Mechanical aortic and mitral valve	stroke, renal failure	Histopathology	vancomycin, gentamicin, rifampicin, doxycycline	Cardiac transplant	This study

Figure legends

Figure 1. Positive immunohistochemistry against *Coxiella burnetii*

Immunohistochemistry against *Coxiella burnetii* was performed as previously reported [3] (up: x100, bottom: x200).

Figure 2. Phylogenetic tree of the *Moraxella* 16S rRNA genes

Sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy and ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed on 1348 residues using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites (of 0.621) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=0.375). Reliability for internal branch was assessed using the aLRT test (SH-Like). Branches with support values <50% were collapsed.

Figure 3. Western blot analysis of the patient serum.

1 μg of Coxiella burnetii, *Moraxella osloensis* CSURP3830, Moraxella bovoculi DSM2114, *Moraxella caprae* DSM19149 or *Moraxella lacunata* P1120 antigens were separated by SDS-PAGE, electro-transferred to a PVDF membrane and incubated with a patient serum sample (collected in 06/2013) at a dilution of 1/100. The presence of antibodies against bacterial antigens was then revealed by incubation with a peroxidase-conjugated anti-human goat antibody and a chromogenic reaction using DAB. Markers of molecular mass (kDa) are indicated on the left.

Figure 4. *M. osloensis* specific PCR on the valve sample

(A) PCR positive control (random-amplified nucleic acids extracted from the positive microdissection area) (B) PCR on the nucleic acids extracted from the microdissection positive area (C) PCR on the nucleic acids extracted from another valve section of the same paraffin bloc, (D) PCR on the nucleic acids extracted from a frozen mitral vegetation. All experiments have been performed with an extraction negative control (processed simultaneously) and a PCR negative control. MW: molecular weight. All the PCR products have been Sanger sequenced and confirmed as *M. osloensis*.

Figure 1

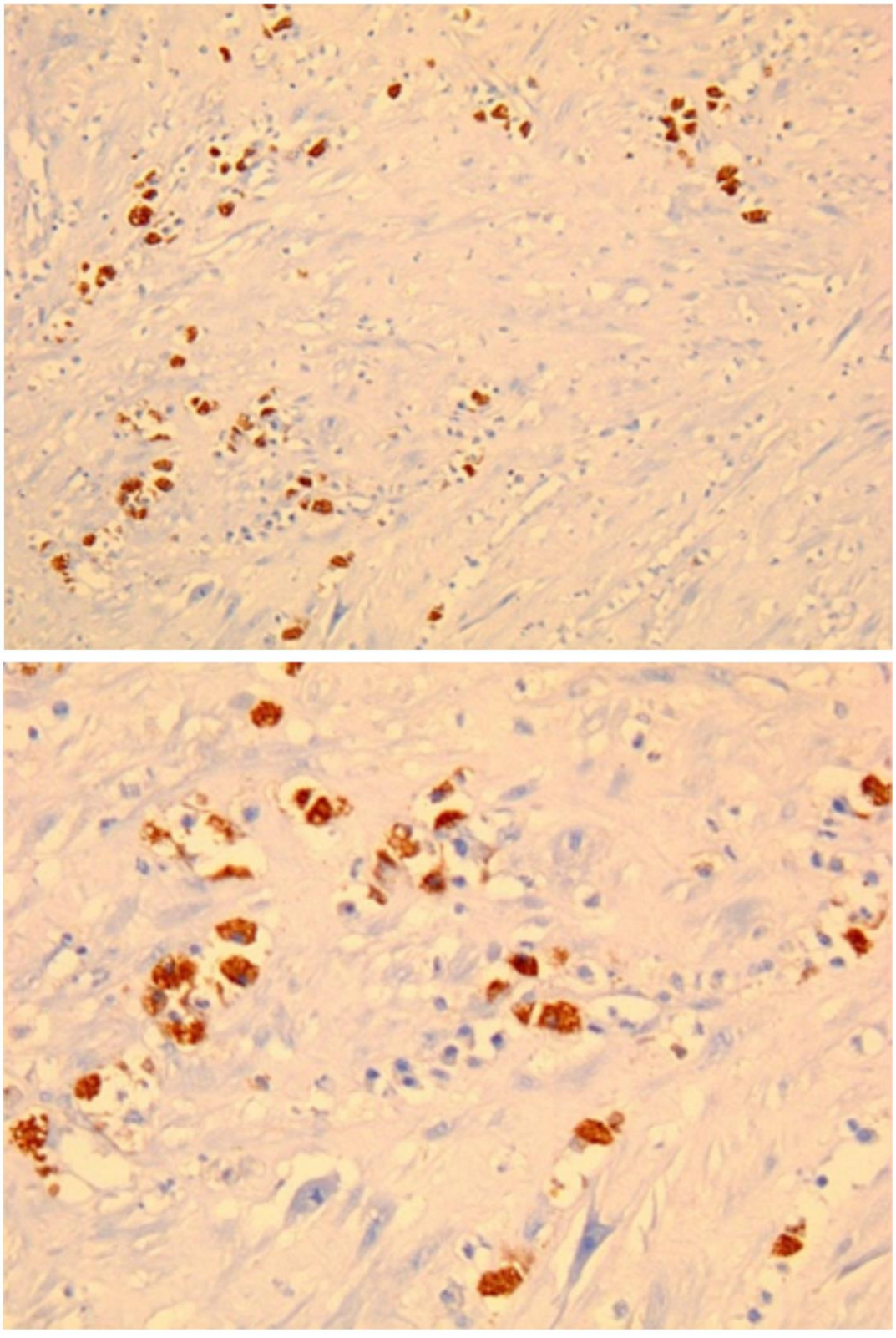
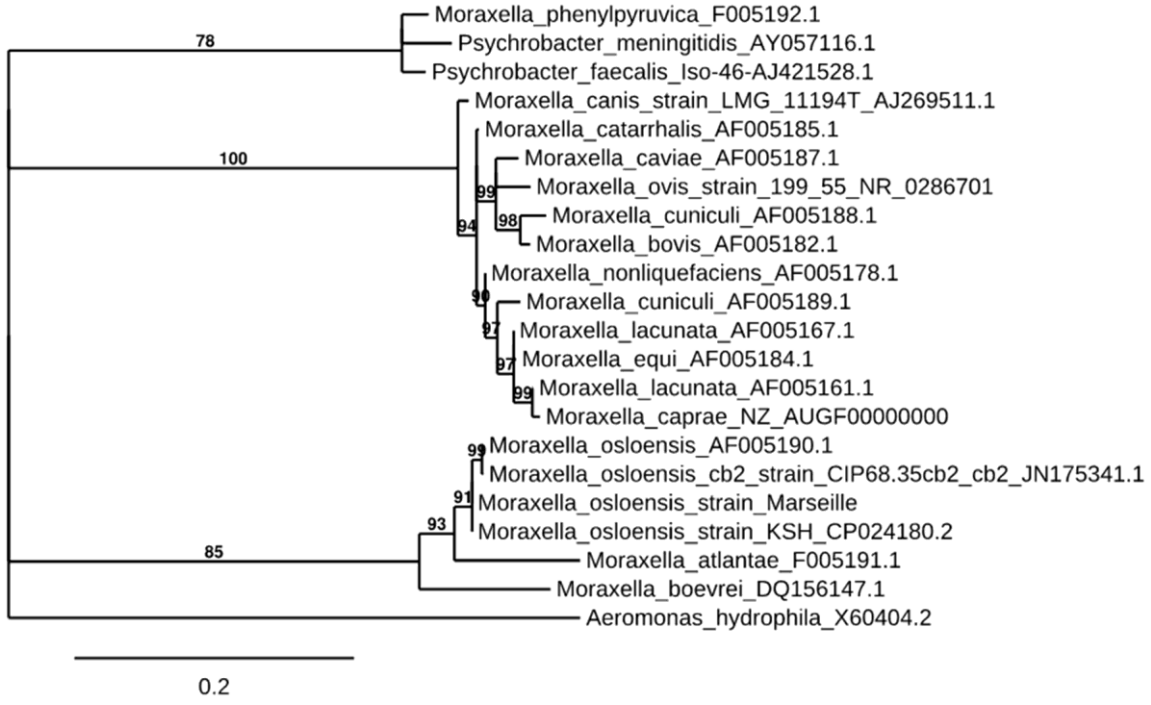


Figure 2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Psychrobacter_faecalis_Iso-46-AJ421528.1
Moraxella_canis_strain_LMG_11194T_AJ269511.1
Moraxella_catarrhalis_AF005185.1
Moraxella_caviae_AF005187.1
Moraxella_ovis_strain_199_55_NR_0286701
Moraxella_cuniculi_AF005188.1
Moraxella_bovis_AF005182.1
Moraxella_nonliquefaciens_AF005178.1
Moraxella_cuniculi_AF005189.1
Moraxella_lacunata_AF005167.1
Moraxella_equi_AF005184.1
Moraxella_lacunata_AF005161.1
Moraxella_caprae_NZ_AUGF00000000
Moraxella_osloensis_AF005190.1
Moraxella_osloensis_cb2_strain_CIP68.35cb2_cb2_JN175341.1
Moraxella_osloensis_strain_Marseille
Moraxella_osloensis_strain_KSH_CP024180.2
Moraxella_atlantae_F005191.1
Moraxella_boevrei_DQ156147.1
Aeromonas_hydrophila_X60404.2
78
100
99
98
94
90
97
97
99
99
91
93
85
0.2

Figure 3

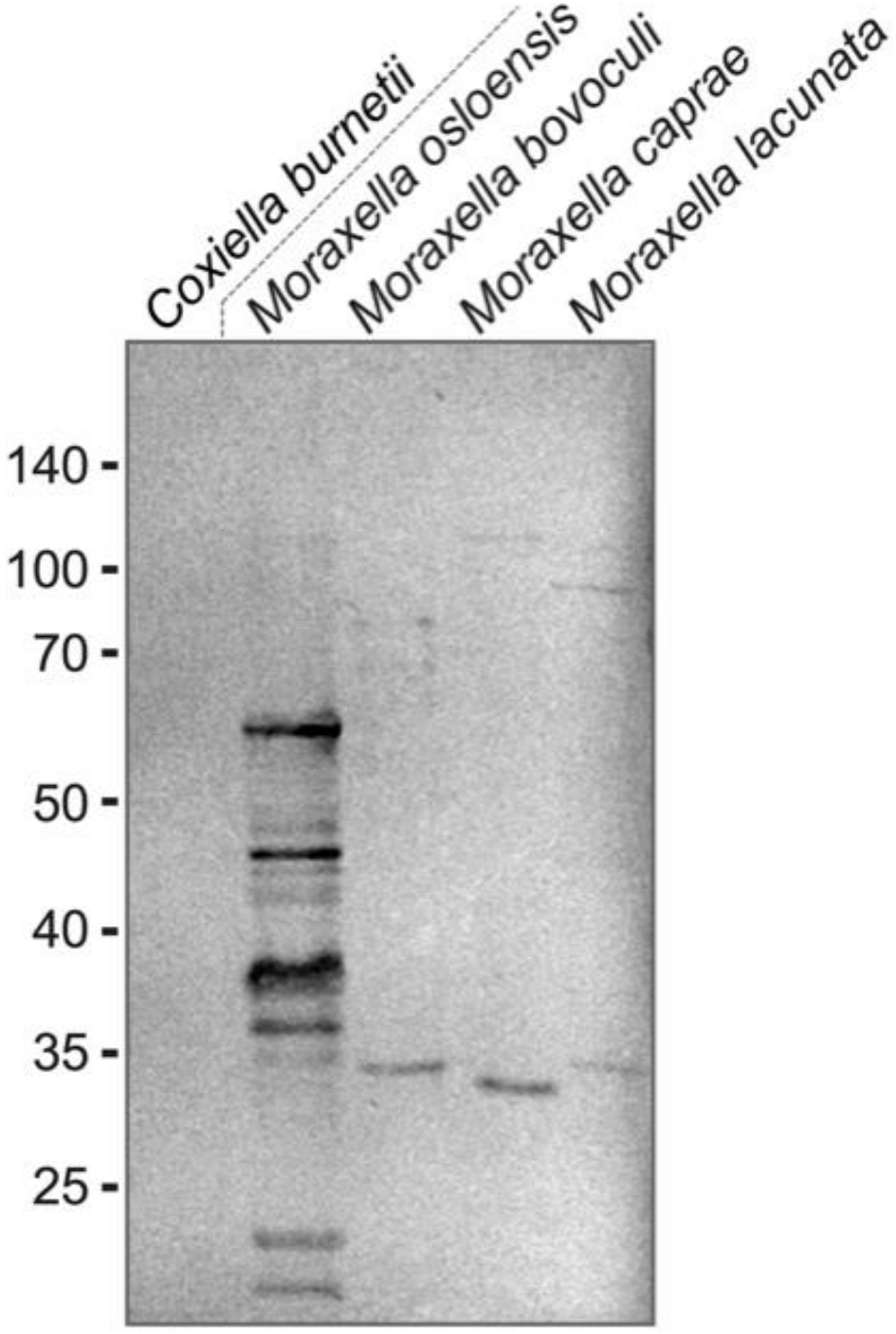
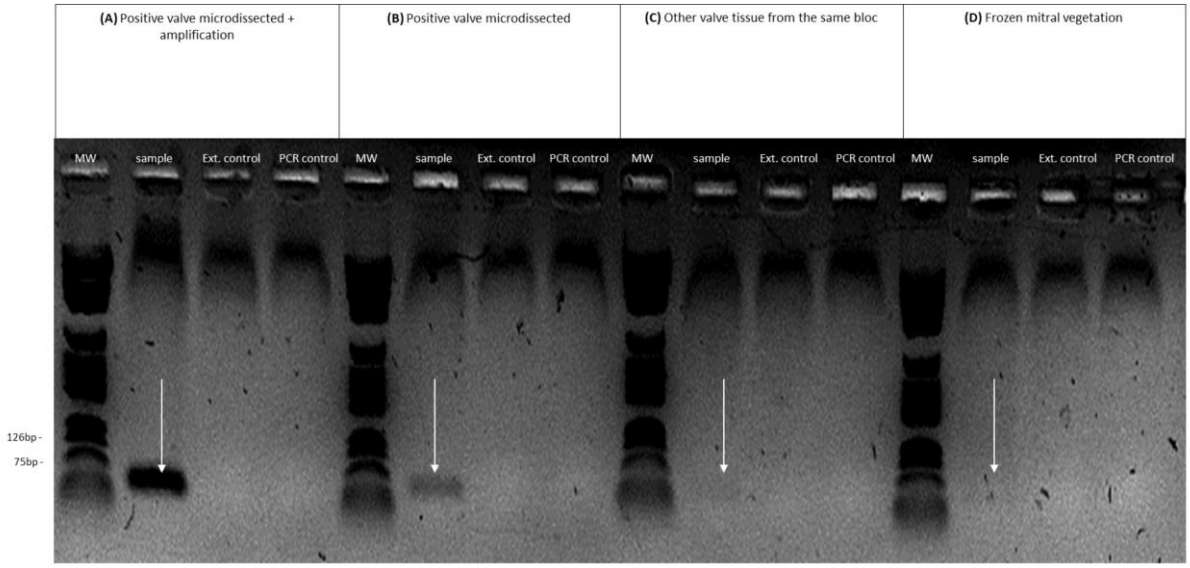

Coxiella burnetii
Moraxella osloensis
Moraxella bovoculi
Moraxella caprae
Moraxella lacunata
140
100
70
50
40
35
25

Figure 4


(A) Positive valve microdissected + amplification
(B) Positive valve microdissected
(C) Other valve tissue from the same bloc
(D) Frozen mitral vegetation
MW
sample
Ext. control
PCR control
126bp -
75bp -