New species for EU aquaculture

Deliverable Report

Deliverable No:	D24.16	**Delivery Month:**	58
Deliverable Title	Report of the major bacterial and viral diseases found in meagre, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided.		
WP No:	24	**WP Lead beneficiary:**	P1. HCMR
WP Title:	Fish Health - Meagre		
Task No:	24.7	**Task Lead beneficiary:**	P2. FCPCT
Task Title:	Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in meagre.		
Other beneficiaries:	P2. FCPCT		
Status:	Delivered/	**Expected month:**	57
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Objective: Report of the major bacterial and viral diseases found in meagre, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided: a compilation will be done of the potential bacterial/viral diseases in meagre related with annual seasonality. Diagnosis tools of the different bacterial/viral diseases of natural occurrence in the meagre will be presented. In addition, the deliverable will include a detailed description of the symptoms found after challenge test against the most common pathogens, as a tool for diagnosis of those diseases. All those results are compiled as a diagnosis manual for the major bacterial and viral diseases found in meagre and protocols for the implementation of useful treatments.

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1. Introduction

Within the last years, a compendium of the fish aquaculture diseases in Spain has been established taking into account the different geographical regions and the different species cultivated within each region. This classification of the different relevant pathologies was done taking into account the potential impact for the aquaculture sector, and the relevance of the different pathogens (Richard and Furones, 2013). Canary Islands has been reported to have a lower incidence of fish pathogens than other regions from the Spanish mainland, highlighting the incidence of opportunistic bacteria such as *Photobacterium piscicida* and *Vibrio spp*., and the occurrence of nodavirus in European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*) has been reported suggesting they are especially susceptible to Nodavirus, but meagre? was not included in the studies from canary Islands included in the report cited above.

2. Major bacterial and viral diseases found in Gran Canaria within the project period.

Within the different years, only few pathology incidences were detected in meagre and are listed in **Table 1**. All samples were seeded in (BHIB) supplemented with 1.5% NaCl at 25 ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl and the bacteria grown were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16S RNA.

Table 1. Occurrence of different pathogens within the project period.

Year	Pathogen species	Outbreaks	
2014	*Nocardia sp.*	No Outbreaks	routine isolation
2015	*Vibrio algynoliticus*	No outbreaks	Routine isolation
2016	*V. algynoliticus*	September 2016	3 broodstock died
	Bacillus sp.	November 2016	mortality occurrence in 23g juveniles
2017	*V. algynoliticus*	No outbreaks	routine isolation
2018	*Photobacterium damselae* subsp. *piscicida*	August 2018	massive mortality in fry (0.2 g body weight)

Some bacteria isolation was also done but only with routine samples (no outbreaks) and only from one or two fish during the whole period (**Fig. 1**). These bacteria were not considered as “major” bacteria.

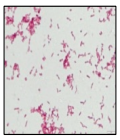
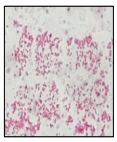
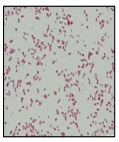
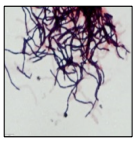
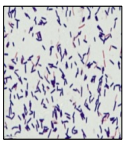
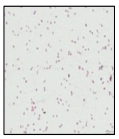
Organ	Spleen	Kidney	Liver			Spleen
	1	2	3	4	5	6
morphology	Gram -	Gram -	Gram -	Gram +	Gram +	Gram -
Foto Gram						
Dx	*Vibrio spp.*	*Aeromonas spp.*	*Vibrio spp.*	*Nocardia spp.*		*Aeromonas spp.*

Figure 1. Some bacteria isolated from different organs of meagre juveniles. Those bacteria were not considered as "major bacteria" for meagre as were only isolated from one fish in routine samples.

However, Nocardia was studied in a challenge test (see below) due to the importance of this bacterial infection in this species, and Bacillus was studied in the context of a minimum inhibitory concentration (MIC) analysis conducted with different antibiotics, as they were the most relevant pathogens found. Fish affected by *Bacillus sp.* showed some ulcerative lesions in skin and? fins, damage to the eyes and fins, and general cachexia (**Fig. 2**).

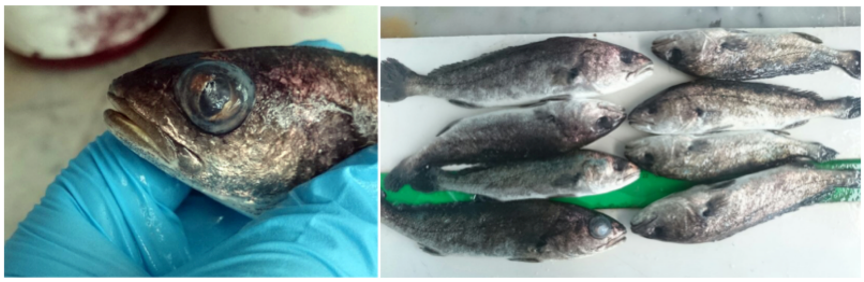


Figure 2. General morphology of meagre juveniles affected by *Bacillus* sp. during November 2016. Fin erosion, eye damage and general cachexia could be easily observed in the affected animals.

The most important mortalities of this species are associated to cannibalism during larvae and fry stages. In addition, the incidence of systemic granulomatosis has also been reported, this information being included in a different deliverable.

Taking into account the susceptibility of this species to Nodavirus infection, 3 routine samples per year were conducted within the P2 facilities, and Nodavirus determination on meagre broodstock within facilities was also conducted with negative records of Nodavirus in the whole population.

For Nodavirus the detection in meagre populations was done by PCR. The Nodavirus (NODA) primers used for detection are given in **Table 2** and were used at a concentration 10 µM (Invitrogen).

Table 2. Primers used to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
NODA 1 reverse	CGAGTCAACACGGGTGAAGA	427 bp
NODA 2 forward	CGTGTCAGTCATGTGTCGCT	

Amplification was performed in a 25 µl volume containing 5 µl cDNA, 2.5 µl 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of $MgCl_2$, 1 µl of each primer, 0.125 µl Taq polymerase (Bioline) and the rest of DEPC water to a total volume of 25 µl for each reaction. The protocol followed is described in **Table 3**.

Table 3. Protocol cycles for the second phase.

TEMPERATURE ºC	TIME	Nº OF CICLES
94	4'30''	1
94	30''	35
58	30''	
68	30''	
68	10'	1

The PCR products were stored at 4 ° C or -20 ° C until separated in agarose gel (2%) electrophoresis. A Nested-PCR for this virus was applied to the PCR products. To this end the pair of internal primers that amplify the PCR product of primers obtained from Table 1 and which generate a amplification product of 159 bp (**Table 4**) was used, at a concentration of 50 mM (Invitrogen).

Table 4. Primers used for a Nested-PCR to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
R31 reverse	AGTGTCTCCAGCTTTCTTCT	159 Bp
F21 forward	GATTTCGTTCCATTCTCTTG	

Amplification was performed in 25 µl volume, containing 3 µl of Nodavirus PCR product, 2.5 ml µl of 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of $MgCl_2$, 1 µl l of each primer, 0.125 ml µl of Taq polymerase (Bioline) and the rest of DEPC water to a total volume of 25 µl, per reaction.

The results obtained are showed in **Fig. 3**: No nodavirus was found in FCPCT meagre population.

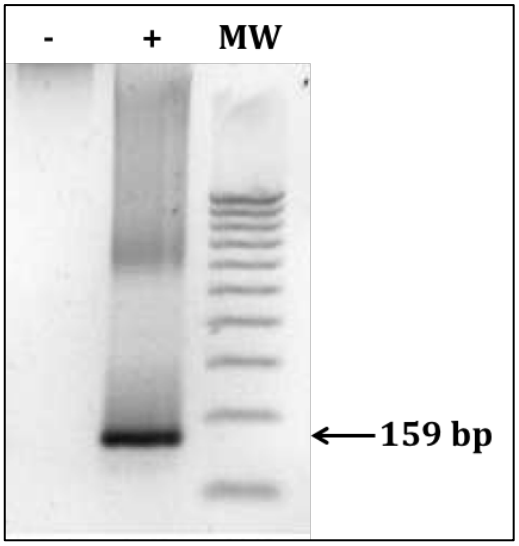


Figure 3. Amplification of Nodavirus PCR product.
Positive control for 159 base pairs.

Samples of different animals from the FCPCT facilities were analysed. From broodstock, samples from nervous system from those broods dead during the project and frozen were analysed. From juveniles, samples of nervous system from previously stressed animals (250 g body weight) were taken after killing with an anaesthetic overdose. The results show a population of virus-free broodstock (**Fig. 4**), and a population of juveniles with no presence of virus (**Fig. 5**). Hence no nodavirus is present in the FCPCT meagre population.

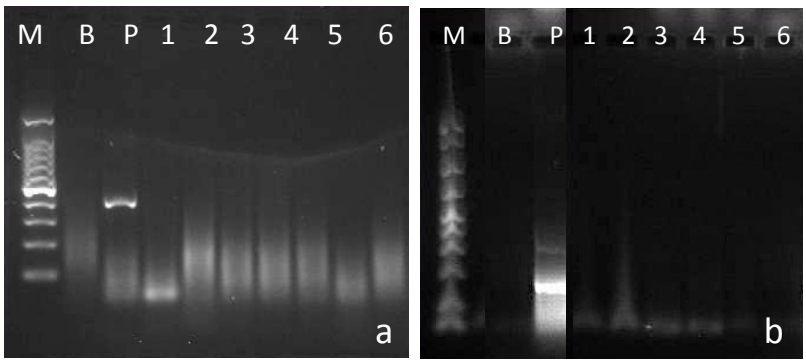


Figure 4. Analysis of nervous system samples from broodstock population a) PCR. b) NESTED- PCR.

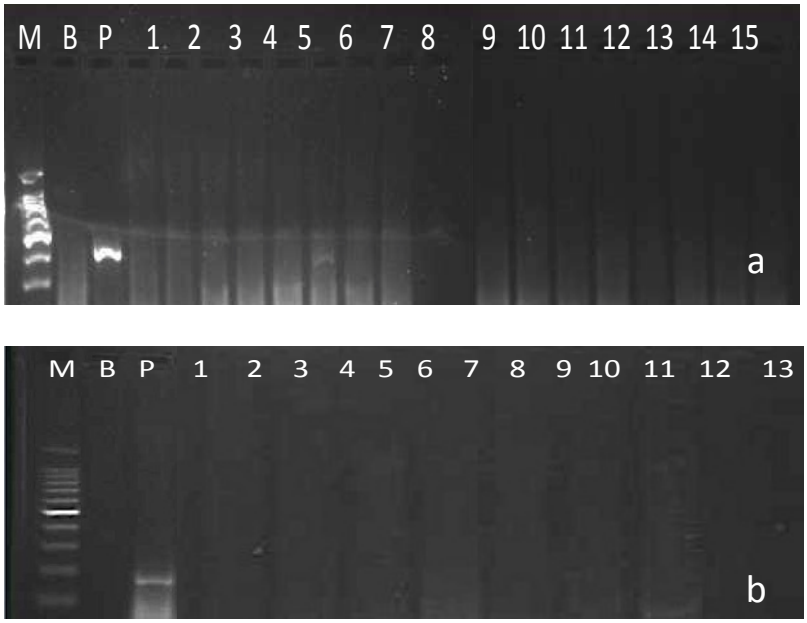


Figure 5. a) Nervous system from Juvenile (PCR). b) Nervous system from juvenile (NESTED- PCR)

3. Experimental challenge tests

Taking into account both the outbreaks/occurrence different challenge test were conducted at the Marine Biosecurity Station from FCPCT (University of Las Palmas de Gran Canaria).

3.1. **Challenge test against *Nocardia sp*.** A challenge test was conducted with the isolated *Nocardia*. A total of 180 fish with an average initial weight of 69.50 g were randomly placed into nine groups, so that 20 fish per group were housed in each 500 mL-tank. They were inoculated by intraperitoneal injection with 1 mL of bacterial suspension with different concentrations of bacteria, from 10^1 to 10^8 CFU/mL, except the control group which was inoculated with sterile phosphate buffered saline (PBS). Inocula were prepared from a culture in YEME medium (broth) supplemented with 0.1% Tween 80, in order to reduce the typical formation of clumps by *Nocardia* spp. After incubating and centrifuging the culture, bacteria were suspended in PBS to get an optical density of approximately 0.5 at 600 nm to finally prepare serial dilutions.

Due to the chronic nature of this disease, three samplings for microbiological and histological analysis were carried out after 30, 37 and 51 days post infection (dpi), so 4 fish per tank and time point were randomly selected to be sacrificed (**Fig. 6).** Also, spontaneously dead fish were recorded daily and then, analyzed as described below for all sampled fish.

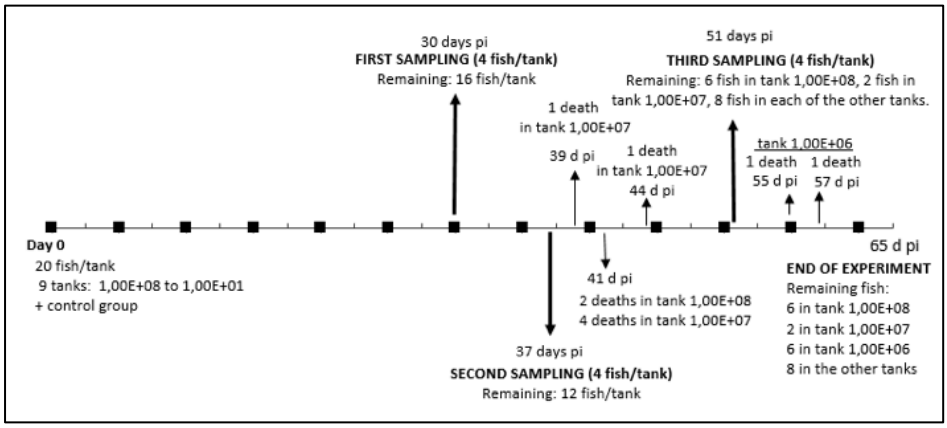


Figure 6. Timeline of an experimental infection in meagre by *Nocardia spp*., with the number of fish sacrificed per time point and spontaneous deaths observed.

Bacterial recovery was from liver, spleen and kidney sections onto blood agar (5% defibrinated sheep blood (v/v)) supplemented with 1.5% sodium chloride (DSBA+1.5%NaCl), brain-heart agar with 1.5% sodium chloride (BHIA+1.5%NaCl) and YEME medium (agar). Cultures were incubated at 25ºC for 3 weeks, but checked daily; then, positive colonies? were confirmed by nested-PCR.

Samples of the liver, spleen and kidney were fixed in 10% buffered formalin and processed for paraffin sectioning to evaluate possible histological findings or lesions due to infection, and stained with haematoxylin and eosin (H&E) and other special stains: Gram stain and modified Ziehl-Neelsen´s staining method (ZN).

The Results obtained showed unexpectedly, no? typical gross signs, such as necrosis and ulceration on skin or nodules in internal organs were visible. Lethargy was observed in the second half of the challenge as were weight discrepancies between animals inoculated with 10^8-10^6 CFU/mL and the remaining groups, inoculated with lower doses. Nonetheless, microscopic granulomas were found during histological evaluation, as shown below (See Histopathology).

Regarding mortality rate, a total of 10 dead fish were found only in groups with the higher doses (10^8-10^6 CFU/mL). The first case was recorded at 39 days post infection and deaths peaked at 41 dpi and stopped at 57 dpi. When the inoculated dose was 10^6 CFU/mL, dead fish appeared at the end of the experimental period (**Fig. 7**).

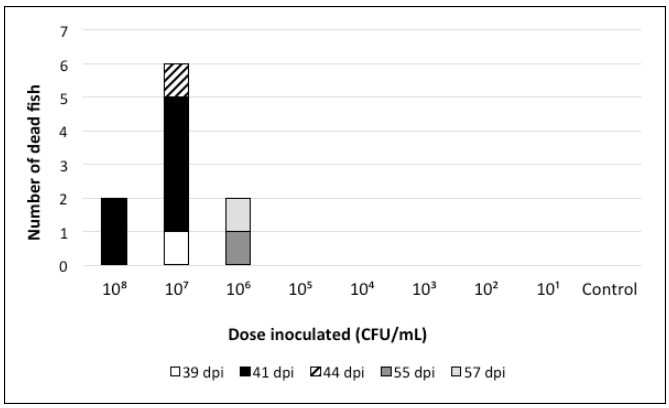


Figure 7. Deaths registered throughout an experimental infection in meagre with *Nocardia brasiliensis*. Inoculated doses from 10^1 to 10^8 CFU/mL.

The cumulative mortality associated to the *Nocardia* challenge test was 17% for doses above 10^6 and 0% at concentrations below 10^6 CFU/mL (**Fig. 8**).

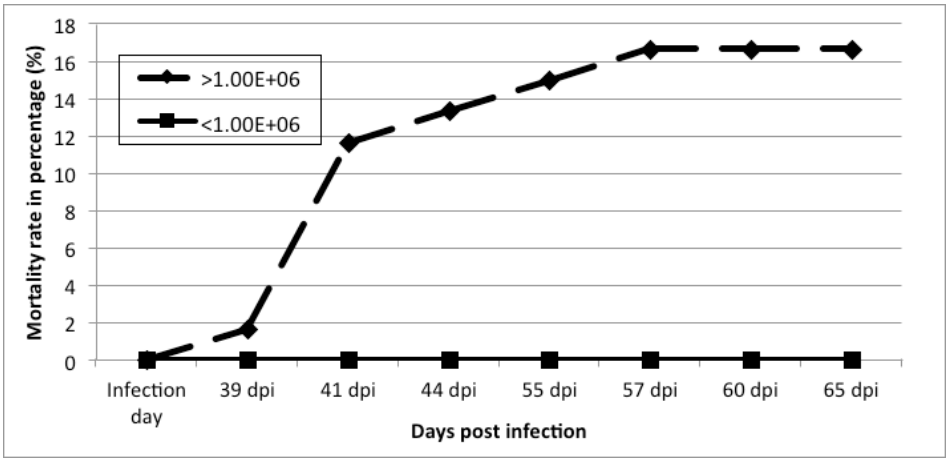


Figure 8. Cumulative mortality of meagre infected with *Nocardia brasiliensis* at concentrations above and below 10^6 CFU/mL.

N. brasiliensis was isolated at the first sampling point (30 dpi) from 50% fish inoculated with a dose of 10^8 CFU/mL and 10^7 CFU/mL, and from 25% of fish injected with 10^4, 10^3 and 10^2 CFU/mL. At the second sampling point (37 dpi), the bacterium was recovered from ~50% and 100% of fish inoculated with doses of 10^8 and 10^7 CFU/mL, respectively (**Fig. 9**). It was possible to isolate the bacteria from the organs of dead fish up to 41 days post infection. From that moment on, *Nocardia* spp. was not detected. Bacteria recovery was higher from liver (11%) than from spleen (4%) and kidney (4%), during the first month relative to the last period, and in the groups "10^8" and "10^7" (**Fig. 9**).

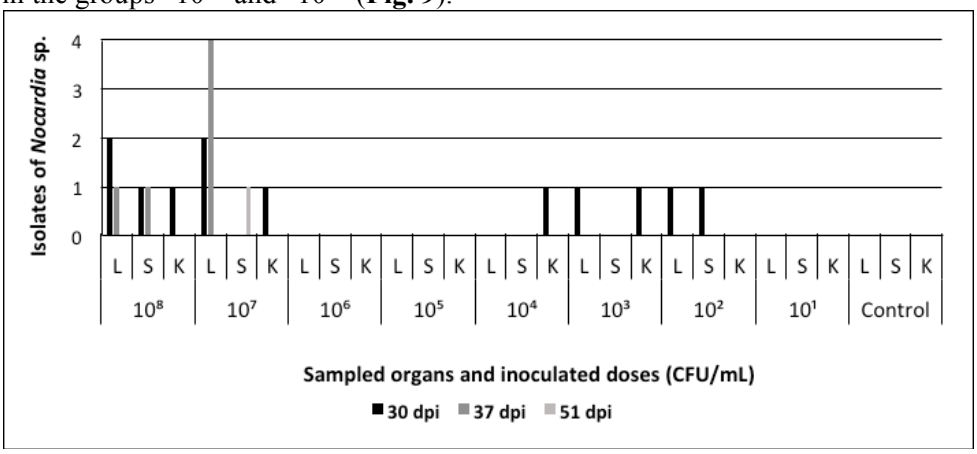


Figure 9. Number of experimentally infected meagre from which *Nocardia brasiliensis* was isolated. Isolates from collected fish organs per experimental group at each sampling point. Abbreviations: L, liver; S, spleen; K, kidney.

Microscopic granulomas were observed from 30 days post infection in internal organs of 25% of fish injected with 10^8, 10^6, 10^5 and 10^4 CFU/mL, reaching 50% of fish with granulomas in the groups inoculated with bacterial concentrations of 10^3 and 10^2 CFU/mL. Then, the incidence of granulomas increased

irregularly throughout the experiment and between doses (**Fig. 10**). It was observed that cases where only one granuloma per organ was visible represented 88%, while only 11% had more than one granuloma. 89% of the animals with more than one granuloma per organ were inoculated with 10^8 and 10^7 CFU/mL. In dead fish, microscopic granulomas were observed in all organs sampled at 41 days post infection (all fish from tanks with 10^8 and 10^7 CFU/mL), but in one? case found two days before, at 39 dpi, no granuloma was observed. Kidney was the tissue most affected with the development of granulomas (26%), followed by liver (12%) and then spleen (3%) (**Fig. 10**).

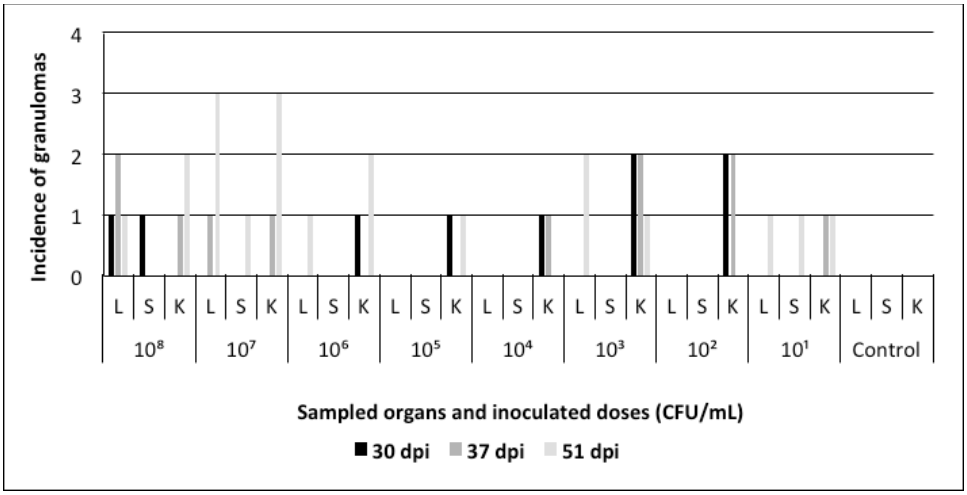


Figure 10. Fish with microscopic granulomas in collected organs at the sampling points of a challenge in meagre with *Nocardia brasiliensis*. Abbreviations: L, liver; S, spleen; K, kidney.

Histologically, there were several types of granulomas: composed by aggregated macrophages (**Fig. 11a**), with macrophages arranged in concentric layers (**Fig. 11b**), with a necrotic center and layers of macrophages (**Fig. 11c**), a larger necrotic center with an external layer of fibrocytes (**Fig. 11d** and **Fig. 11e**) and, the least expected type, completely composed of laminar material without a necrotic center (**Fig. 11f**).

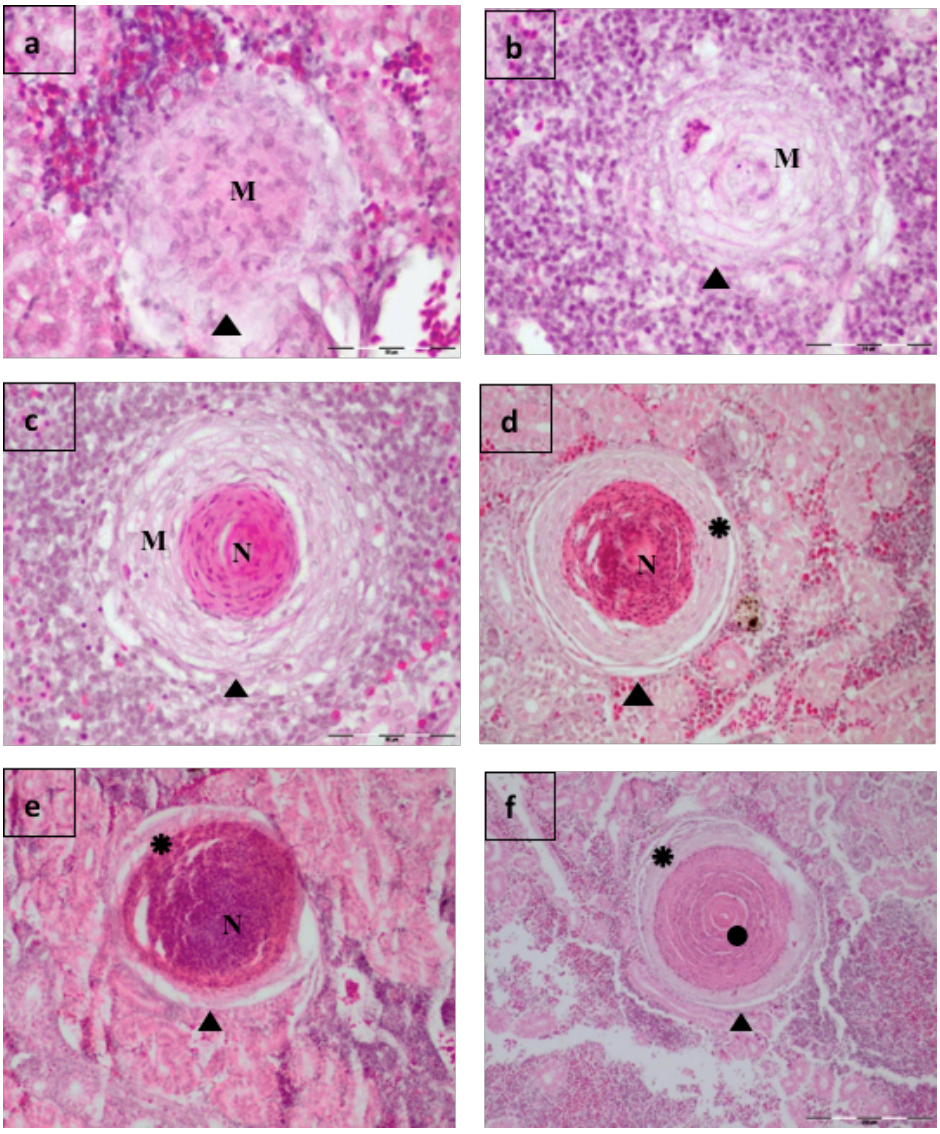


Figure 11. Histopathology of nocardiosis in meagre. Different types of granulomas: (**a**) aggregates of macrophages form the granuloma, (**b**) similar to the first but the cells are arranged in concentric layers, (**c**) with necrotic center, (**d**) composed by necrotic center and surrounded by layers of macrophages and an outer layer of fibrocytes, (**e**) like the previous granuloma without the layers of macrophages, (**f**) a different pattern of granuloma development, composed completely of laminar material. Symbols: ▲, granuloma; **M,** macrophages**; N,** necrotic center; *, layer of fibrocytes; •, laminar material. Magnifications: a-d: x40; e-f: x20. Scale bars: a-c: 50 µm; f: 200 µm.

All granulomas were found in kidney, liver and spleen (**Fig. 12a,b,c**) and most of them were thought to represent different stages of development, despite all types being observed at any dose and at any time.

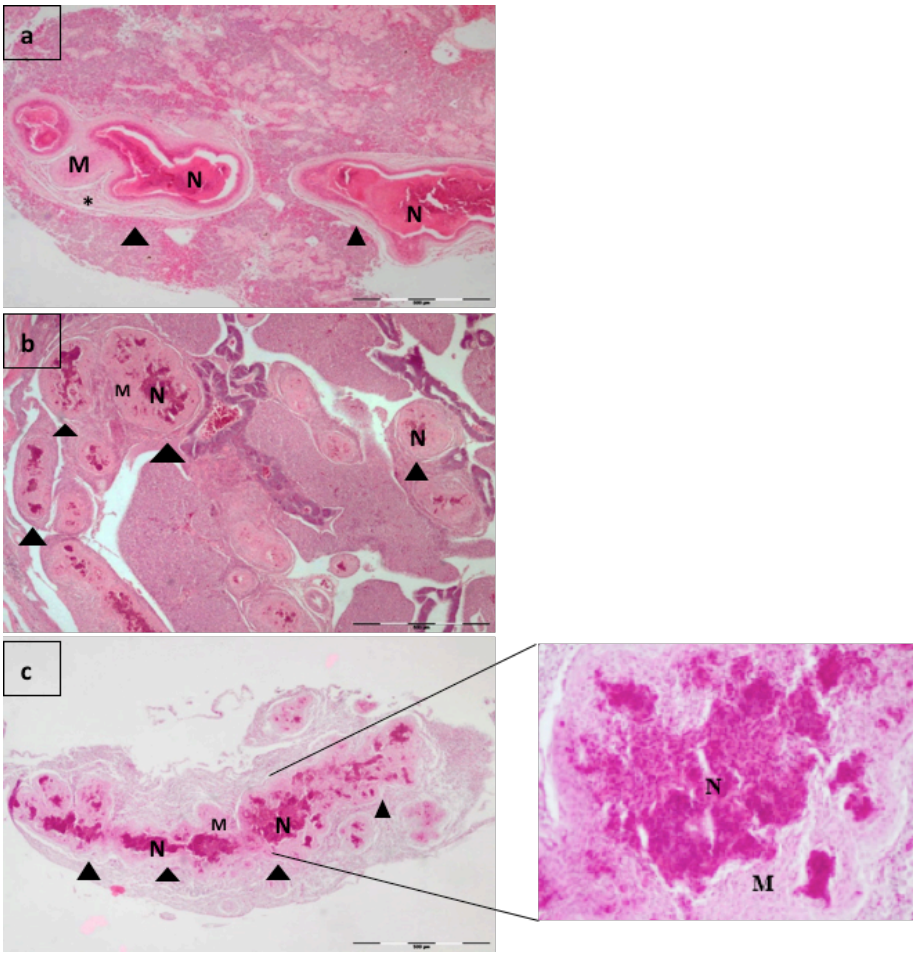


Figure 12. Histopathology of nocardiosis in meagre. Presence of granulomatous foci (▲) in (a) kidney, (b) liver and (c) spleen (with a magnification from the marked area), varied in size with marked necrotic centers (**N**), surrounded by macrophages (**M**) and connective tissue (*). Magnification: x4. Scale bar: 500 µm.

Moreover, we could not find any bacteria in histological sections, even with special stains (**Fig. 13**).

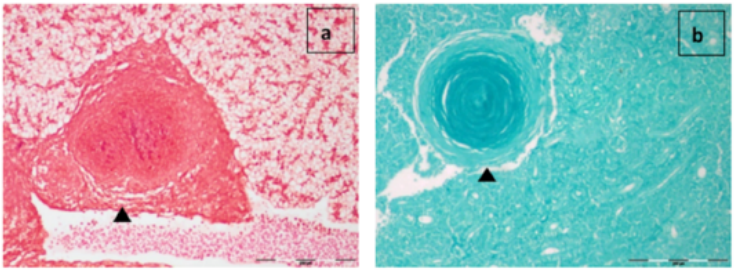


Figure 13. Special stains, (a) Gram and (b) Ziehl-Neelsen, did not reveal the presence of *Nocardia spp*. in histological sections. ▲, granuloma. Magnification: x10. Scale bar: 200

3.2. Challenge test against Photobacterium damselae and Vibrio anguillarum. Forty meagre juveniles (average 40 g body weight) obtained from FCPCT fish stock, were maintained in a closed seawater flow circuit with water at a temperature of 22ºC and a salinity of 37‰ under a 12-h light/12-h dark cycle. The fish were intraperitoneally inoculated with 100 µl? of the different bacterial strain *Photobacterium damselae subsp. piscicida* ME-1 or *Vibrio anguillarum* at 10^5 colony-forming units/fish. Those bacteria were isolated from clinical outbreaks registered in Canary Islands. Inoculated and control fish were monitored for clinical disease and mortalities for the duration of the study (15 days). Mortality was attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs (**Fig. 14**). Only *Photobacterium damselae* subsp. *piscicida* produced mortality in meagre juveniles.

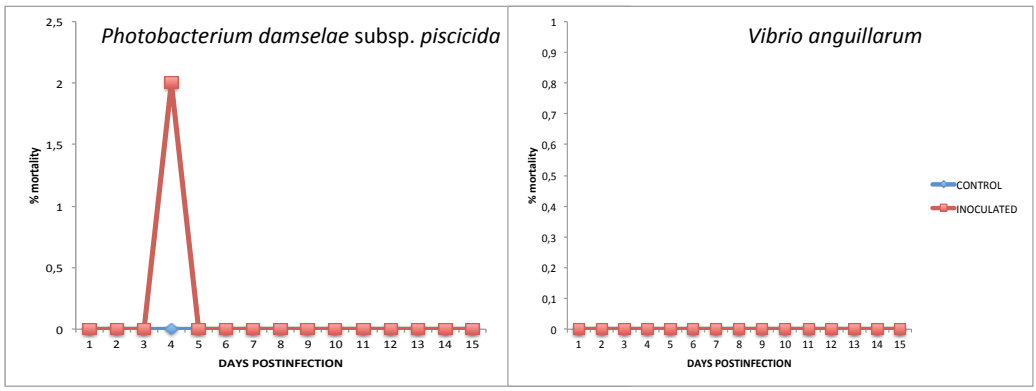


Figure 14. Results of challenge test against different bacteria isolated from outbreaks registered in farms from Canary Islands.

4. Minimum inhibitory concentration (MIC) of different antibiotics for the major bacteria in meagre.

Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline (Sigma-Aldrich) were the tested antibiotics. The initial concentrations used varied from 1280µg/ml to 10µg/ml for all the antibiotics except for Sarafloxacin, which was used from 800µg/ml to 6.25µg/ml, and Oxytetracycline, used from 1000µg/ml to 7.8125µg/ml, in serial dilutions with a dilution factor of 1:2. The antibiotics were diluted in TSB when used for *Vibrio anguillarum*, *Bacillus sp.*, *Vibrio alginolitycus* and *Photobacterium damselae subsp. piscicida*. The total volume used for the incubation in the microtiter plate was of 100µl, 50µl from bacteria and 50µl from the solution of antibiotic. The experiment was repeated x3 for every bacteria and antibiotic. The turbidity of the suspensions on the microtiter plate was measured by spectrophotometry. The measurements were made at a wavelength of 600nm.

Results of MIC for *Vibrio anguillarum* (**Fig. 15**) showed that the isolated strain of meagre presents MICs for erythromycin of 80µg/ml, for florfenicol of 5µg/ml, for oxytetracycline of 0.898µg/ml and for sarafloxacin of 0.781 µg/ml.

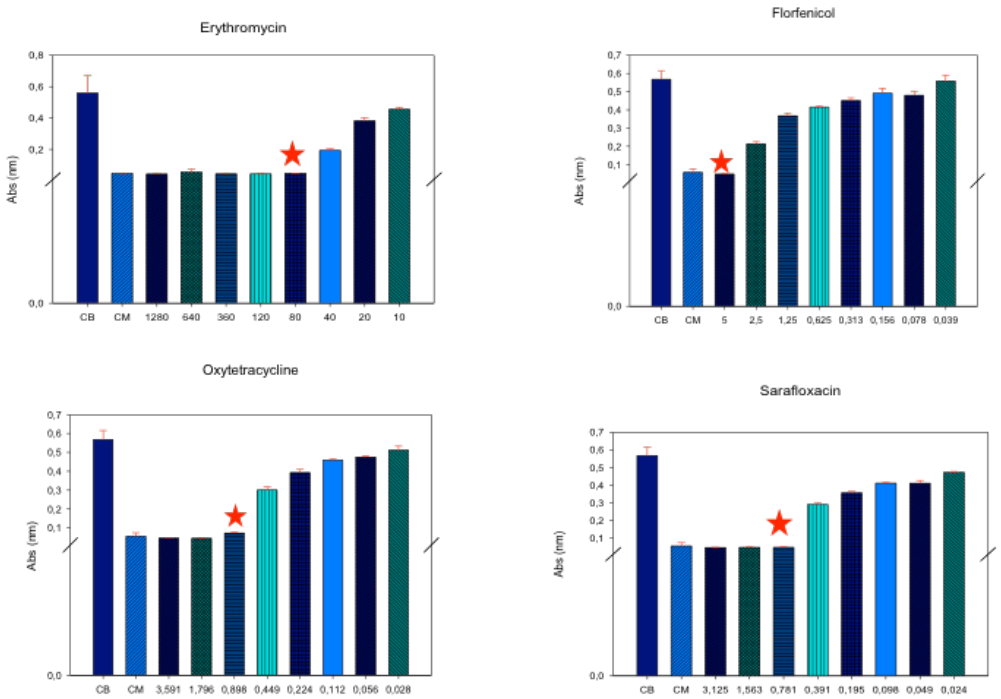


Figure 15. Absorbances obtained with respect to the concentration of antibiotic used for *Vibrio anguillarum*. The sample marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Bacillus sp.* (**Fig. 16**) showed that the isolated strain of meagre presents MICs for erythromycin of 160µg/ml, for florfenicol of 10µg/ml, for oxytetracycline of 1.25µg/ml and for sarafloxacin of 0.78 µg/ml.

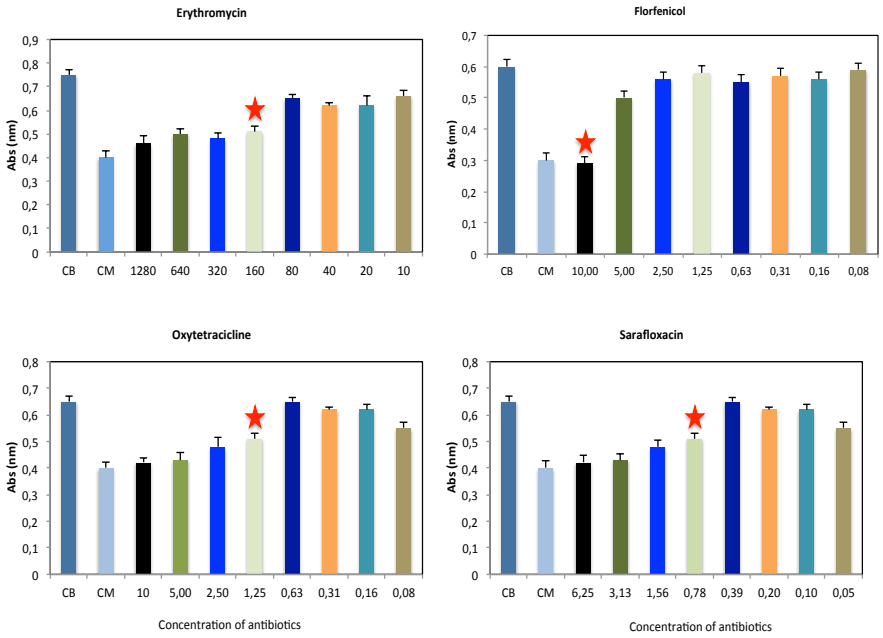


Figure 16. Absorbances obtained with respect to the concentration of antibiotic used for *Bacillum sp*. The sample marked with the star represents the MIC for the tested antibiotic

Results of MIC for *Vibrio algynolyticus* (**Fig. 17**) showed that the isolated strain of meagre presents MICs for oxytetracycline of 3.591µg/ml. This strain was resistant for the rest of the antibiotics tested.

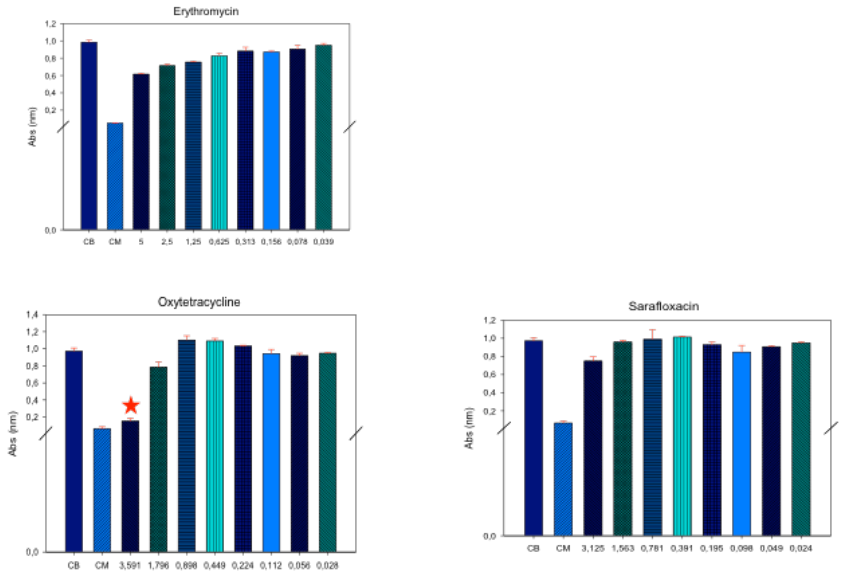


Figure 17. Absorbances obtained with respect to the concentration of antibiotic used for *Vibrio algynolyticus*. The sample marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Photobacterium damselae subsp. piscicida.* (**Fig. 18**) showed that the isolated strain of meagre presents MICs for erythromycin of 80µg/ml, for florfenicol of 5µg/ml, for oxytetracycline of 250µg/ml but was resistant for sarafloxacin.

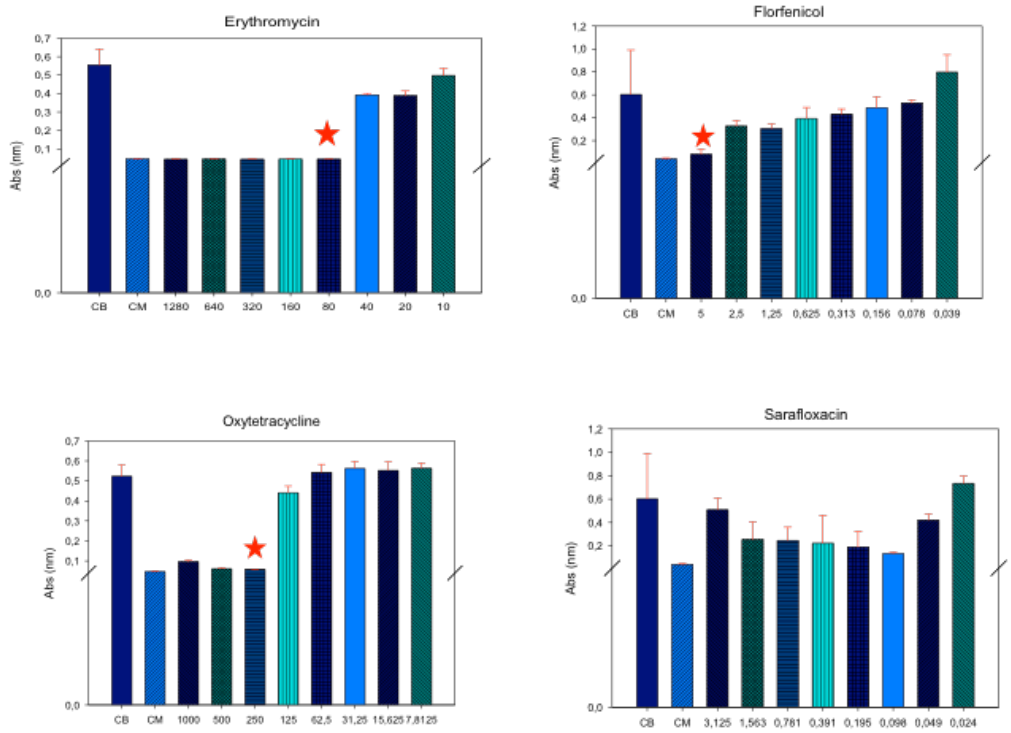


Figure 18. Absorbances obtained with respect to the concentration of antibiotic used for *Photobacterium damselae* sub. *piscicida*. The sample marked with the star represents the MIC for the tested antibiotic

With regard to isolates in meagre, we observed a constant sensitivity to oxytetracycline with MICs from 250 to 0.898 µg/ml, which seems to be the antibiotic of choice in the treatment of these pathologies as a general treatment against bacteriosis. The rest of the antibiotics had resistance from some isolates and their use would require prior identification.

5. Recommended protocols

5.1. Molecular protocols for diagnosis by PCR

PCR And QPCR Tuning For The Diagnosis Of Pathogens

Bacterial and viral strains have been obtained from our collection. The bacteria have been grown overnight in liquid BHI at 22ºC and the viruses have been cultured in different cell lines. The DNA of the bacteria was extracted with a Wizard® Genomic DNA Purification Kit (Promega) and the RNA of the viruses with the a Total RNA Kit I (E.Z.N.A., OMEGA), following the protocol established by the manufacturer.

The pathogens that we detected by PCR (**Fig. 19**) are:

• *Vibrio anguillarum:*

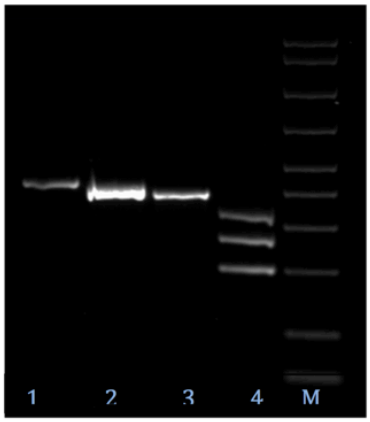
The sequences of the primers used are: VAFW 5'-ACA TCA TCC ATT TGT TAC-3 '(Forward) and VARV 5'-CCT TAT CAC TAT CCA AAT TG-3' (Reverse). These primers amplify a 429 bp fragment.
The PCR reaction is performed in a total volume of 25 µl, containing 2.5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.2 µM of each primer, 0.2 µg DNA, 0.5 U Taq DNA polymerase (Gen Script) and MiliQ sterile water to complete the total volume.
The amplification protocol consists of an initial denaturation at 95ºC for 10 min, 25 cycles of denaturation at 95ºC for 30 s, hybridization at 56ºC for 30 s, extension at 72ºC for 30 s, and a final extension at 72ºC for 7 min. After PCR amplification, 2 µl of the product is analyzed on a 1.5% agarose gel.
The PCR reaction is performed in a total volume of 50 µl, containing 5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.5 µl DNA from each Vibrio, 1 U Taq DNA polymerase (Gene Script) and sterile MiliQ water to complete the total volume.
The amplification protocol consists of an initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, hybridization at 60ºC for 30 s, extension at 72ºC for 120 s, and a final extension at 72ºC for 10 min. After PCR amplification, 2 µl of the product is analyzed on a 2% agarose gel.

• Multiplex of *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*:

The sequences of the primers used are: VALFW 5'-GAG AAC CCG ACA GAA GCG AAG-3 '(Forward) and VALEV 5'-CCT AGT GCG GTG ATC AGT GTT G-3' (Reverse), VPFW 5 ' -GAA AGT TGA ACA TCA TCA GCA CGA-3 '(Forward) and VPRV 5'-GGT CAG AAT CAA ACG CCG-3' (Reverse) and VVFW 5'-TTC CAA CTT CAA ACC GAA CTA TGA-3 '(Forward) and VVRV 5'-ATT CCA GTC GAT GCG AAT ACG TTG-3 '(Reverse). These primers amplify a fragment of 337 bp, 271 bp and 205 bp, respectively.
The PCR reaction is performed in a total volume of 50 µl, containing 5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.5 µl DNA from each Vibrio, 1 U Taq DNA polymerase (Gene Script) and sterile MiliQ water to complete the total volume.
The amplification protocol consists of an initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, hybridization at 60ºC for 30 s, extension at 72ºC for 120 s, and a final extension at 72ºC for 10 min. After PCR amplification, 2 µl of the product is analyzed on a 2% agarose gel.

• *Photobacterium damselae subsp. piscicida:*

The sequences of the primers used are: PDSP FW 5'-AGG GGA TCC GAT TAT TAC TG-3 '(Forward) and PDSP RV 5'-TCC CAT TGA GAA GAT TTG AT-3' (Reverse). These primers amplify a fragment of 410 bp.
The PCR reaction is performed in a total volume of 25 µl, containing 2.5 µl 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.2 µg DNA, 1.25 U Taq DNA polymerase (Gen Script) and MiliQ sterile water to complete the total volume.
The amplification protocol consists of an initial denaturation at 94 ° C for 5 min, 35 cycles of denaturation at 94 ° C for 30 s, hybridization at 55 ° C for 30 s, extension at 72 ° C for 1 min, and a final extension at 72 ° C for 7 min. After PCR amplification, 2 µl of the product is analyzed on a 1.5% agarose gel.



1- *Vibrio anguillarum*. 429 pb.
2- *Photobacterium damselae* subsp. *piscicida*. 410 pb.
3- *Vibrio harveyi*. 382 pb.
4- Multiplex for *Vibrio alginolyticus* (337 pb), *Vibrio parahaemolyticus* (271 pb) & *Vibrio vulnificus* (205 pb).
M- Molecular weight marker, top to bottom:2000 pb, 1500 pb, 1000pb, 700 pb, 500 pb, 400 pb, 300 pb, 200 pb, 100 pb, 50 pb.

Figure 19. PCR for the selected pathogens assayed

• *Nocardia*

The identification of the bacterial genus was performed by a Nested Polymerase Chain Reaction (Nested PCR) analysis. Deoxyribonucleic acid (DNA) was extracted using a Genomic DNA Extraction Kit (Promega) and the Nested PCR protocol followed in this work was based on the one described by Laurent *et al.* (1999; corrected in 2000) and subsequently upgraded by Elkesh *et al*. (2013), although with some more modifications, namely the initial conditions were as described by Remuzgo (2014, pp.51-52). In the first reaction, a set of universal primers conserved in all 16S rRNA was used: forward primer PA (5´-AGAGTTTGATCCTGGCTCAG-3´) and reverse primer PL06 (5´-GCGCTCGTTGCGGGACTTAACC-3´), obtaining an amplicon of 1000 bp which undergoes a second run with a pair of primers, NG1 (forward) (5´-ACCGACCACAAGGGGG-3´) and NG2 (reverse) (5´- GGTTGTAAACCTCTTTCGA-3´), designed to amplify a Nocardia genus-specific 16S rRNA gene fragment and give a product at 600 bp (**Fig. 20**).
Then, the first PCR product was sent to the Research Institute IDIVAL (Santander, Spain) for bacterial identification by sequencing the amplified 16S rRNA gene fragment.

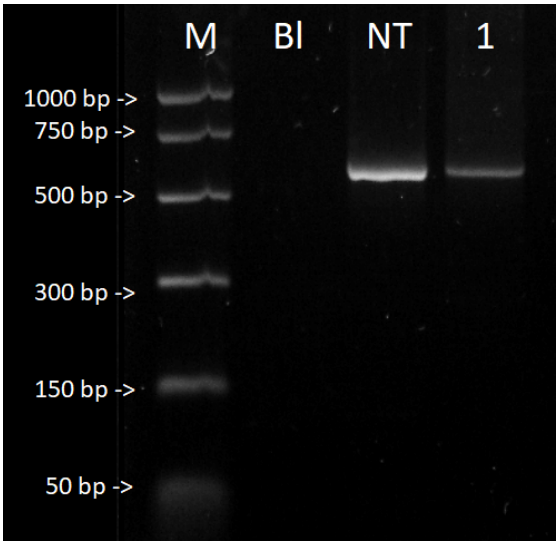


Figure 20. Electrophoresis on 1.5% agarose gel with gel red staining after nested-PCR of *Nocardia spp*. Expected band size= 600 bp. M, molecular weight marker (Promega); Bl, blank; NT, *Nocardia asteroides* CECT 3051; 1, Nocardia *spp.* isolated from meagre (*Argyrosomus regius*).

5.2. Recommended protocols to industry

Based on the results obtained of the occurrence of different pathogens and experimental challenge test the following recommendations on specific antibiotic plus dosage and protocols can be formulated. It could be of interest to point out that it is necessary to sample moribund fish and perform PCR diagnosis for the main pathogens of the meagre. Depending on the result, the established treatments are:

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Vibrio anguillarum	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the sole ration for 5 consecutive days	**RECOMMENDED**
	Sarafloxacin	10-15 mg/kg	Single dose	
	Erythromycin	0.1 g/kg fish	Feed as the sole ration for 21 consecutive days	

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Bacillus sp	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	
	Sarafloxacin	10-15 mg/kg	Single dose	**RECOMMENDED**
	Erythromycin	0.1 g/kg fish	Feed as the single dose for 21 consecutive days	

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Vibrio algynolyticus	Florfenicol		-	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	**RECOMMENDED**
	Sarafloxacin		RESISTANT STRAIN	
	Erythromycin		RESISTANT STRAIN	

BACTERIA	ANTIBIOTIC	DOSE	ORAL ROUTE	
Photobacterium damselae subsp. piscicida	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	**RECOMMENDED**
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	
	Sarafloxacin		RESISTANT STRAIN	
	Erythromycin	0.1 g/kg fish	Feed as the single dose for 21 consecutive days	

Deviation from DOW

No deviation from the plan.

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