REVIEW ARTICLE

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Photobacterium damselae subsp. *piscicida*: an integrated view of a bacterial fish pathogen

Abstract Pasteurellosis, or pseudotuberculosis, is a bacterial septicaemia caused by the halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*). Although this disease was first described in wild populations of white perch and striped bass, currently the natural hosts of the pathogen are a wide variety of marine fish. The disease has great economic impact both in Japan, where it affects mainly yellowtail cultures, and in the Mediterranean area, due to the losses it causes in seabream and seabass farms. This microorganism serves as a perfect model to study a bacterial fish pathogen, either at an applied level, to resolve or to mitigate the high economic losses of fish farmers, or at a basic level, for a better understanding of *P. damselae* subsp. *piscicida* biology. This article discusses the methods employed in our laboratory to study the causative agent of pasteurellosis. It reviews important aspects, from the diverse procedures for the detection and isolation of the pathogen to the latest molecular studies that have allowed its correct taxonomic allocation. Characterization of some virulence mechanisms and the available methods to prevent the disease are also presented.

Keywords *Photobacterium damselae* subsp. *piscicida* · Taxonomy · Virulence · Detection · Vaccine · Fish pathology

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Introduction

Pasteurellosis is a bacterial septicaemia also referred to as pseudotuberculosis because, in chronic cases, diseased fish show, in several internal organs, whitish tubercles consisting of bacterial accumulations. The halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) is the aetiological agent of pasteurellosis. Due to this change in the taxonomic position of the causative agent, some authors have renamed this septicaemia as photobacteriosis. However, to avoid possible confusion with other fish diseases caused by other species of the genus *Photobacterium*, this review will maintain the name pasteurellosis.

The disease was first described in wild populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963, when a massive epizootic occurred in Chesapeake Bay (USA) [52]. Sniezsko and coworkers placed the microorganism isolated during this first epizootic within the genus *Pasteurella* on the basis of its morphological and biochemical properties. Later, Jansen and Surgalla [11] examined the microorganism and found sufficient distinctions in physiological and serological characteristics from other species in the genus to propose the new species name *Pasteurella piscicida*. A few years later, pasteurellosis became a problem in yellowtail (*Seriola quinqueradiata*) and ayu (*Plecoglosus altivelis*) cultures in Japan [13, 16, 17,34]. Soon thereafter, the disease spread to other fish species in Japan, and several epizootics were described in red seabream (*Acanthopagrus schlegeli*), red grouper (*Epinephelus akaara*), oval file fish (*Navodan modestus*), and hybrid striped bass [*M. saxatilis* (*M. chrysops*)] [see review 28].

In Europe, isolation of the bacterium from rudd (*Scardinius erythrophthalmus*), chub (*Coregonus zenithicus*), Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), or sheat fish (*Silurus glanis*) was reported in different countries such as England, Norway and Hungary, but the infectious agent was later confirmed as

Aeromonas salmonicida isolates. No cases of pasteurellosis were noted on the continent until 1990; Toranzo et al. [55] described the first outbreak of the disease in Spain, which affected juvenile gilthead seabream (*Sparus aurata*) cultures in the northwestern area. Almost simultaneously, outbreaks of pasteurellosis also occurred in southwestern Spain, France, Italy, Israel, Greece and Portugal, mainly in populations of seabream and seabass [see review 28]. Since then, pasteurellosis has been a major limiting factor in the culture of these fish species in the Mediterranean region.

Today, the natural hosts of the pathogen are a wide variety of marine fish. Pasteurellosis has had a great economic impact in Japan, where it affects mainly yellowtail cultures, and in the Mediterranean area, because of the losses it causes in seabream and seabass farms. For these reasons, many aspects of the disease and its causative agent *P. damselae* subsp. *piscicida* have been extensively studied, resulting in a quite complete picture of fish pasteurellosis. This article reviews the current state of knowledge of *P. damselae* subsp. piscicida, focusing on important aspects such as the phenotypic and genetic characterization of the bacterium, its main virulence mechanisms, and effective strategies to prevent the disease.

Isolation and phenotypic characterization of isolates

P. damselae subsp. *piscicida* can be isolated from internal organs of diseased fish after 2–4 days incubation at 22 °C by using media such as trypticase soy agar (TSA) and blood agar, both supplemented with 1–2% NaCl, or marine agar 2216E. The presumptive diagnosis is based on the isolation of a gram-negative, non-motile bipolar rod that is oxidase- and catalase-positive, fermentative without gas production, sensitive to the vibriostatic agent O/129, and has strict salt requirements. However, diagnosis can be hampered by the slow growth of this bacterium in laboratory media, which is easily obscured by other fast-growing bacteria. This fact greatly encourages biochemical, serological and genetic characterization studies in order to develop an accurate and specific diagnostic procedure.

Based on such exhaustive characterization, a complete description of the pathogen was obtained [9,28]. *P. damselae* subsp. *piscicida* is a pleomorphic bacterium changing from coccoidal to long rods depending on the culture conditions. The etiological agent of fish pasteurellosis shows positive responses for arginine dihydrolase, lipase and phospholipase activities, and is negative for indole, nitrate reduction, urease, gelatinase, amylase and hydrogen sulfide production [28]. Regarding the use of carbohydrates, this bacterium produces acid only from glucose, mannose, galactose and fructose. Variable results were reported for methyl-red, Voges-Proskauer, sucrose and maltose reactions [41]; as suggested by Koike et al. [15], the employment of different basal media could account for this variability. Hemolytic activities against

trout and sheep erythrocytes were not detected. *P. damselae* subsp. *piscicida* can grow at temperatures from 15 to 32.5 °C, although its optimum growth temperature is 22.5–30 °C. It is able to produce capsular material [1] composed of 99.6% carbohydrate and 0.4% protein. These overall phenotypic results show that *P. damselae* subsp. *piscicida* constitutes an homogeneous group that differs from the other subspecies – *P. damselae* subsp. *damselae* (formerly *Vibrio damsela*) – in important biochemical and physiological traits, such as motility, gas production from glucose, nitrate reduction, urease, lipase, amylase, and hemolysin production [5,24]. The homogeneity of *P. damselae* subsp. *piscicida* facilitates use of the miniaturized system API-20E for its identification. In agreement with Kent [12], although the pathogen is not included in the API-20E code index, the system is valuable for a rapid presumptive identification of the bacterium, because all strains have a similar pattern (2005004), with neither false-positive nor false-negative reactions being detected [47].

From a serological standpoint, *P. damselae* subsp. *piscicida* is also a highly homogeneous group [24,46], which makes it impossible to establish serotypes. Its serological homogeneity was supported by lipopolysaccharide (LPS) profiles. In fact, all strains analyzed, regardless of their origin or source of isolation, had the same electrophoretic pattern: O side-chains consisting of high-molecular-mass bands in a ladder-like pattern [24]. In addition, similar profiles of total and outer membrane proteins (OMPs) were also observed for all isolates. The LPS and OMP profiles of all the *P. damselae* subsp. *piscicida* strains proved to be immunologically related in the respective Western blot assays [24,28]. This antigenic uniformity encouraged the development of serological techniques for the detection and identification of *P. damselae* subsp. *piscicida* isolates. Slide agglutination and latex agglutination tests, direct immunofluorescent assays, and enzyme-linked immunosorbent assay (ELISA) procedures are among the methods that have been employed and even commercialized for this purpose [14, 41, 43,45].

Photobacterium damselae subsp. *piscicida* showed a different plasmid content depending on the strain analyzed. Thus, whereas European and USA strains share plasmids of 20 and 7 MDa, the Japanese isolates have a common 37-MDa plasmid. In addition, a high-molecular-mass plasmid band of approximately 50 MDa has been detected in the majority of European isolates studied [24]. Restriction endonuclease analysis demonstrated that the plasmid bands shared by most European strains are genetically homogeneous.

Genetic studies and the design of molecular detection methods

Despite the fact that pasteurellosis has long been recognized as a disease of fish, the taxonomic position of its etiological agent, *Photobacterium damselae* subsp. *piscicida*, was a matter of contro-

versy that has only recently been clarified. On the basis of its physiological characteristics, such as Gram staining, oxidase activity, absence of motility, and the typical bipolar rod shape, the bacterium was first assigned to the genus *Pasteurella* [52]. Shortly after, Janssen and Surgalla [11] proposed the name *Pasteurella piscicida* to designate this pathogen, although some traits did not match with the description of the genus (i.e. its inability to reduce nitrate reduction and its sensitivity to low pH and temperatures). *P. piscicida* was at that time the only halophilic member of the genus. In the early 1970s, some Japanese authors suggested different genera, such as *Corynebacterium* [13] and *Arthrobacter* [48], as better taxonomic positions for this fish pathogen on the basis of diverse characteristics including carbohydrate metabolism, pleomorphism, and sensitivity to penicillin. Immunological techniques showed that *P. piscicida* had no common antigens either with *Corynebacterium* or with *Arthrobacter* [21]. However, a positive relationship was observed with *Pasteurella plecoglosicida* isolated from ayu, which supported the first assignation of the bacterium to the genus *Pasteurella*.

It has been reported that *P. piscicida* shares major phenotypic characteristics with other fish pathogens including *Vibrio anguillarum*, *Aeromonas salmonicida*, and the clinically important pasteurellae, *Pasteurella multocida* and *Pasteurella haemolytica* [24]. A study of the fatty acid methyl ester (FAME) profiles by gas chromatography [44] revealed significant homogeneity in the major fatty acids among the different *P. piscicida* strains. In addition, comparison of these results with those obtained from representative strains of *V. anguillarum*, *A. salmonicida* and *Pasteurella* species of clinical origin suggested that the agent of fish pasteurellosis should be closer to *Pasteurella* than to *Vibrio* or *Aeromonas*.

On the other hand, De Ley et al. [2], employing DNA-rRNA hybridization techniques, suggested that *P. piscicida* should be a member of the *Vibrionaceae* strongly related to *V. parahaemolyticus*. Unfortunately, this study included only a presumptive isolate of *P. piscicida*, which, according to the culture conditions specified by the authors, could be the result of cross-contamination. Later, Nicolas et al. [36], on the basis of rRNA studies, reallocated *P. piscicida* as a new subspecies of *Vibrio damsela*. Due to this long uncertainty and controversy, the name *Pasteurella piscicida* was never included in the Approved List of Bacterial Names [50]. In 1994, Ruimy et al. [8] carried out an exhaustive study of the family Vibrionaceae and related organisms by means of 16S rRNA sequencing and DNA-DNA hybridization. One year later [9], they proposed the reassignment of *P. piscicida* as a new subspecies of the recently created *Photobacterium damsela* comb. nov. (formerly *Vibrio damsela*)[51], since the two organisms differ only in one nucleotide in the 16S rRNA sequence and showed a DNA-DNA hybridization of approximately 80%. These results were later confirmed by other authors [39], who also demonstrated the high similarity in rRNA sequence of other members of the genus *Photobacterium* (98.1% for *P. histaminum*) with both subspecies of *P. damsela*. Therefore, the new, and finally approved, name for the causal agent of fish pseudotuberculosis was established as *Photobacterium damsela* subsp. *piscicida*. Truper and De'Clari [56] corrected the specific epithet "damsela" (substantive) to "damselae" (genitive) giving the current name of the bacterium, *Photobacterium damselae* subsp. *piscicida*.

Once the taxonomic position of the bacterium responsible of fish pasteurellosis had been clarified, and new molecular procedures for genetic analysis became available, studies to obtain better knowledge of the pathogen's genome were carried out. Ribotyping and random amplification of polymorphic DNA (RAPD) showed the existence of two clonal lineages within *P. damselae* subsp. *piscicida* (Fig. 1), one including the European strains and the other the Japanese isolates [31, 33,53]. These results constituted the first evidence of genetic heterogeneity within this fish pathogen.

In addition, the number of sequenced genes from *P. damselae* subsp. *piscicida* has increased in recent years, not only to further characterization of the bacterium, but also to find a sequence on which the design of a subspecies-specific detection method could be based. The high degree of similarity in the 16S rRNA sequence of
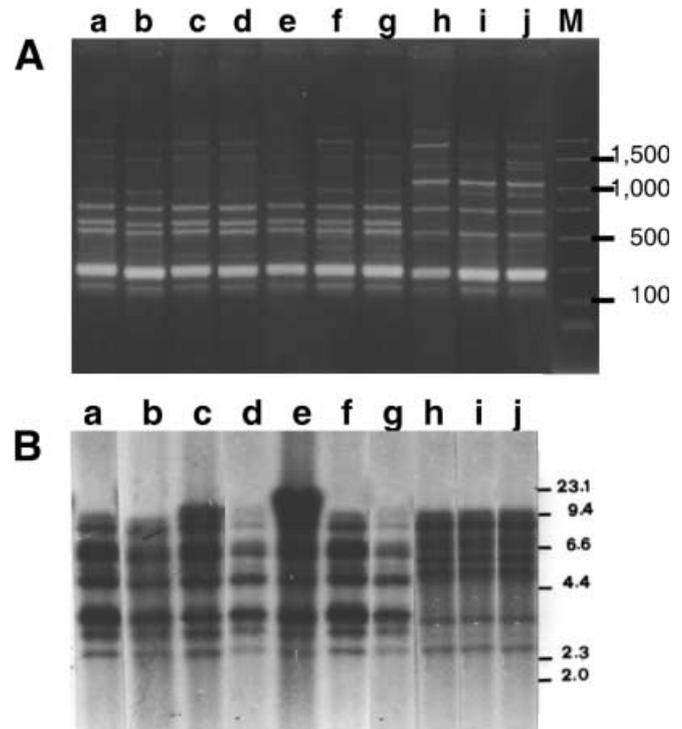

Fig. 1. RAPD fingerprints (**A**), achieved after DNA amplification using primer 4 (Amersham Pharmacia), and ribotype patterns (**B**), obtained employing the endonuclease PvuII, for the *P. damselae* subsp. *piscicida* isolates. *Lanes*: *a–g* European isolates, *h–j* Japanese isolates, *M* molecular ladder (50–2,000 bp; Bio-Rad). The molecular sizes (in kb) are indicated on the *right*

the two subspecies of *P. damselae* has allowed only the development of a PCR-based detection method at a species level [39]. We have compared the sequences of the two other ribosomal genes, 5S and 23S, in both subspecies [38] and found similarities also higher than 99%. The intergenic spacer regions ITS-1 (located between the 16S and 23S genes) and ITS-2 (between the 23S and 5S genes), which are believed to evolve between lineages at a higher ratio than rRNA genes, showed again similarity percentages of 98–99.5%. Note that these genomic regions have a peculiar mosaic-like structure, and there is a possibility that there is some inter-subspecies heterogeneity in terms of differential reorganizations of the sequence pieces [38].

The gyrB gene, coding for the B subunit of the DNA gyrase, as well as four regulatory genes, toxR, rpoN, luxS, and a sigma-54-dependent activator, were also analyzed as alternative molecular chronometers. In all cases, the results supported those deduced from the ribosomic operon study, i.e. high similarities between both subspecies [38].

Furthermore, we investigated at a DNA level one of the main phenotypic differences between *P. damselae* subsp. *piscicida* and subsp. *damselae* [40], namely, urease activity. Whereas subsp. *damselae* harbors the *ureC* gene, included in the *ure* operon, subsp. *piscicida* lacks this gene, which is in accordance with its urease-negative phenotype. Although these results raise new questions on the speciation processes and the dramatic phenotypic divergence of two subspecies so closely related at the ribosomal operon level, they were decisive for the design of a subspecies-specific detection method. This method, based on multiplex-PCR for 16S rRNA and *ureC* genes [40], is currently the only PCR assay that can successfully discriminate between subspecies of *P. damselae* (Fig. 2), and is therefore a serious candidate to become a useful tool for diagnosis of pasteurellosis in the field. In fact, the assay is being used in our laboratory for routine detection of *P. damselae* subsp. *piscicida* (unpublished results).

Fig. 2. Agarose electrophoresis of the multiplex-PCR products obtained for the different *Photobacterium* strains studied. *Lanes*: M, Molecular mass markers (100-bp molecular ladder; Bio-Rad); a–d, *P. damselae* subsp. *damselae* strains; e, *P. histaminum* isolate; f–p *P. damselae* subsp. *piscicida* strains. Numbers on the *right* indicate the size of the amplification products, corresponding to the 448- and 267-bp internal fragments of the *ureC* and 16S rRNA genes, respectively

Fig. 3A, B. Transmission electron micrographs of thin sections of cells of *P. damselae* subsp. *piscicida*. **A** Avirulent strain EPOY-8803-II; **B** virulent strain DI

Virulence mechanisms

Photobacterium damselae subsp. *piscicida* is a highly pathogenic bacterium that does not seem to have host specificity. Therefore, pasteurellosis can be a risk even for marine fish species in which the disease has not been yet described. Some authors have pointed out differences in susceptibility of some fish species (i.e. gilthead seabream and seabass) to pasteurellosis on the basis of fish age [37]. Thus, whereas small seabream (less than 5 g) are susceptible to the disease, fish above 50 g become resistant. This is due to the functionality of macrophages and neutrophils in larger seabream, which can efficiently phagocytize and kill the bacteria [37,49]. In any case, the fact that this bacterium is highly pathogenic indicates that it must have strong virulence mechanisms.

Adherence and invasive capacities are essential in the first stages of infection. Although *P. damselae* subsp. *piscicida* has been described as adhering weakly to different cell lines, it showed significant binding to fish intestines, with values ranging from 10^4–10^5 bacteria per gram of tissue [29]. In addition, despite being only moderately invasive, the pathogen has been demonstrated to survive for a few days within host cells and even to spread to adjacent cells [29].

Extracellular products (ECPs) secreted by a variety of bacterial fish pathogens are important virulence factors, since they can contribute to the development of the disease in terms of bacterial nutrition or aggresins, enabling the bacteria to counteract the host's defence mechanisms [54]. The ECPs of *P. damselae* subsp. *piscicida* strains were shown to be lethal for different fish species and for mice, and had phospholipase, cytotoxic and hemolytic activities as major characteristics [25]. Histological studies have implicated these activities, in particular the phospholipases, in the pathogenesis of pasteurellosis [37].

Today it seems clear that the main virulence factors of *P. damselae* subsp. *piscicida* consist of polysaccharide capsular material (Fig. 3) [1, 23, 30, 42] and a high-affinity siderophore-mediated iron-sequestering system.
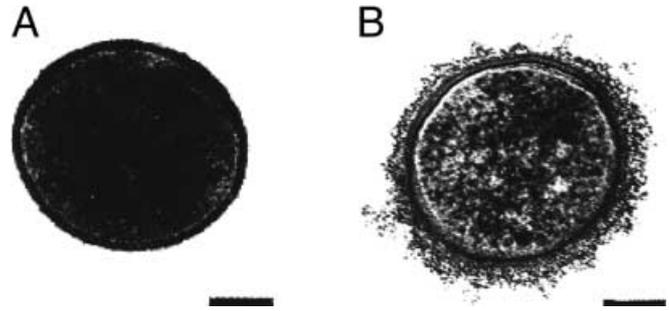
Implication of the capsular material in virulence was clearly demonstrated in experimental fish infections using virulent constitutively capsulated and avirulent non-capsulated forms of *P. damselae* subsp. *piscicida*. The induction of capsular expression in the non-capsulated forms increased their resistance to the bactericidal action of the fish serum and resulted in a 50% lower lethal dose (LD_{50}), around 2–3 log units [30].

The ability to acquire iron is essential for the growth of pathogenic bacteria within the host; therefore, it is also essential to cause infections. The existence in *P. damselae* subsp. *piscicida* of a chromosomally located iron uptake system of has been described. It consists of a siderophore, chemically and biologically related to multicidin produced by *Pasteurella multocida*, and, at least three iron-regulated high-mass OMPs [26]. In addition, it has also been reported that *P. damselae* subsp. *piscicida* can utilize hemin directly as the sole iron source by means of constitutive and additional inducible surface protein receptors, and that intraperitoneal injection of hemin before experimental infection increased the lethality of this pathogen [26].

Further studies [3,4] demonstrated the relationship between both virulence mechanisms. First, it was evidenced that capsular polysaccharides play a minor role in the binding of hemin and, second, that the expression of capsular material is dependent on iron availability and growth phase. Regarding this second point, cells grown under iron-limited conditions always had a significantly lower amount of capsular material than iron-supplemented cells [3]. This finding can be explained by the need of the bacterium to express its siderophore and/ or iron receptors during its time within the host circulatory system. Once the microorganism reaches the different tissues, the amount of capsular material probably increases in response to host cellular defence mechanisms (i.e. phagocytosis by macrophages). In addition, the role of iron in the expression of enzymatic activities has been reported, and some proteolytic enzymes, such as gelatinase and caseinase, are only synthesized when strains are cultured under iron-restricted conditions [26].

It is obvious that the pathogenesis of *P. damselae* subsp. *piscicida* is a complex, multifactorial process not

yet fully understood, although the considerable work carried out in the last few years has contributed to clarifying it to a great extent.

Vaccination: an effective measure of prevention

Chemotherapy was effective for the treatment of fish pasteurellosis until the late 1980s. Since then, plasmid- or chromosome-coded resistance to ampicillin, tetracycline, and other drugs has been observed [25,36]; therefore, new chemotherapeutants, such as florfenicol and phosphomycin, have been employed to control pasteurellosis outbreaks. We have used proteomic analysis to study the mechanisms of resistance. Comparison of resistant and susceptible isolates by two-dimensional polyacrylamide gels and analysis of the protein sequences have yielded promising results which will aid in understanding the biology of resistance acquisition. Moreover, it has been described that *P. damselae* subsp. *piscicida* has a period of intracellular parasitism within macrophages during infection [20], which can explain the ineffectiveness of chemotherapy in the treatment of some outbreaks of the disease. Therefore, immunoprophylaxis has become the best way to prevent pasteurellosis.

Throughout the last 20 years, there have been a variety of studies analyzing the effectiveness of immunization in preventing pasteurellosis [42]. Most vaccines tested consisted of heat- or formalin-killed cells [6, 10, 18,19]. Although some protection was achieved with these preparations, the results were not reproducible [20]. Better results were obtained using formulations based on LPS and ribosomal fractions of the bacteria [7,22]. However, these formulations presented not only problems of reproducibility, but also difficulties of production on a large scale. Passive immunization has also been evaluated [6], but, due to the short period of protection achieved, it was effective only if the vaccine was administered up to 24 h prior to challenge.

The best protection against pasteurellosis was obtained with an ECP-enriched bacterin developed in our laboratory, achieving relative percent survival (RPS) values higher than 75% when vaccinating fish between 0.5 and 2 g [27]. Further studies demonstrated that vaccination at the larval stage (50-day old fish) is also effective, with RPS values of 84–90% [31]. Therefore, the vaccination program recommended consists of a first immunization when larvae are around 50 mg and a booster immunization when fish reach 2 g body weight. This vaccine is currently commercially available and has been successfully employed in several European countries including Spain, Portugal and Greece.

Future trends in vaccine formulations against *P. damselae* subsp. *piscicida* are the use of live attenuated bacteria [19], although their utilization in the field is currently not allowed, and the use of iron-regulated OMPs [26] as protective antigens. Both formulations have shown promising results in preliminary studies.

Conclusions

Great effort has been invested in the last few years in the study of *Photobacterium damselae* subsp. *piscicida*, a bacterium that can serve as a model for the study of any bacterial fish pathogen. Although some aspects of this complex disease and its etiological agent are not yet fully understood, the global extent of this disease and the enormous economic losses it causes will constitute an important impetus to further our knowledge of the pathobiological characteristics of this microorganism.

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Table 1. SEM Biennial Prize. The Spanish Society for Microbiology (SEM) Biennial Prize dates back to 1983, when the SEM decided that a lecture should be given by a young researcher at each SEM National Congress. The nominees are selected from among the SEM membership; they must be under 40 years of age, and carrying out research of excellence in a field of microbiology

The following researchers have been awarded the SEM Biennial Prize (the centers indicated are those where the scientists worked when they received the prize):
First: Juan Ortín Montón, Center for Molecular Biology (CBM), Autonomous University of Madrid (10th SEM National Congress, Valencia, 1985)
Second: Enrique Herrero Perpiñán, Department of Microbiology, University of Valencia (11th SEM National Congress, Gijón, 1987)
Third: Ernesto García López, Center for Biological Research (CIB), CSIC, Madrid (12th SEM National Congress, Pamplona, 1989)
Fourth: Antonio Ventosa Ucero, Department of Microbiology, University of Sevilla (13th SEM National Congress, Salamanca, 1991)
Fifth: Alicia Estévez Toranzo, Department of Microbiology, University of Santiago de Compostela (14th SEM National Congress, Zaragoza, 1993)
Sixth: Sergio Moreno Pérez, Department of Microbiology, University of Salamanca (15th SEM National Congress, Madrid, 1995)
Seventh: Daniel Ramón Vidal, Department of Biotechnology, Institute for Agrochemistry and Food Technology (IATA), CSIC, Valencia (16th SEM National Congress, Barcelona, 1997)
Eighth: José Antonio Vázquez Boland, Department of Animal Pathology, Complutense University of Madrid (17th SEM National Congress, Granada, 1999)
Ninth: Jesús L. Romalde, Departament of Microbiology and Parasitology. University of Santiago de Compostela (18th SEM National Congress, Alicante, 2001)