Novel bacterium and vaccine.

The present invention relates to a Gram-negative pleiomorphic rod-shaped bacterium causing Big Belly disease in fish, to a microbiological culture comprising said bacterium, to a vaccine derived thereof and methods for the preparation of such a vaccine, to antibodies reactive with said bacterium, to diagnostic test kits and to the use of said bacterium.

Over the last decades, world-wide a strong increase is seen in the consumption of fish. This equally regards the consumption of cold water fish such as salmon, turbot, halibut and cod, and tropical fish such as Asian seabass, tilapia, milkfish, yellowtail, amberjack, grouper and cobia.
As a consequence, an increase has been seen in the number and the size of fish farms, in order to meet the increasing needs of the market.

As is known from e.g. animal husbandry, large numbers of animals living closely together are vulnerable to all kinds of diseases, even diseases hardly known or seen, or even unknown, before the days of large-scale commercial farming. This is equally the case in fish farming.

Recently, the inventors observed an unknown disease in juvenile Asian seabass (*Lates calcarifer*) in a commercial hatchery in Indonesia in the region west of Jakarta. Mortality arose typically in early weaned fish at around 25 to 30 days post hatching. At that moment in time, fish had an approximate size of more than 22 mm in length. Initially it was thought that the mortality could be caused by incomplete nutrition of the larvae during the live feed feeding (i.e. rotifers and *Artemia*). Attempts were made to enrich the live feed with essential fatty acids through enrichment of the live feed with squid and cod liver oil. This only resulted in a delay of the onset of mortality, i.e. mortality arose at 40-50 days post-hatching. It did however not decrease the final level of mortality.
It was therefore assumed that the disease was not primarily caused by incomplete nutrition.

Therefore it was decided to further investigate the cause of this disease.

This case showed some resemblance to the following case: in 1997-1998, in a seabass hatchery in Indonesia, a hitherto unknown disease was seen in seabass. The mortality reached 100% within a week, and affected fish were found to have a slightly swollen abdomen and showed sluggish swimming. In the fish, bacteria-like organisms were observed through histological analysis, but it was impossible to grow these organisms, so the cause of the disease remained an unsolved mystery. The case has been reported by Zafran, I.K. et al., in Diseases in Asian Aquaculture IV, C.R. LaviUa-Pitogo & E.R. Cruz-Lacierda (eds.) Fish Health Section, Asian Fisheries Society, Manila (2002). To the best of our knowledge, no further reporting of the disease has been made, let alone that attempts to find the cause of the disease have been initiated.

It is an aim of the embodiments disclosed herein to provide the causative agent of this enigmatic disease as well as vaccines aiming at combating and preventing the disease. Moreover, it is an objective of the present invention to provide means to detect and identify the causative agent.

In a first aspect, there is provided gram-negative pleiomorphic rod-shaped bacterium causing Big Belly disease in fish, characterized in that said bacterium comprises a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1 or characterized in that the bacterium comprises a 16 S rRNA gene that reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/-15 base pairs.

In a second aspect, there is provided gram-negative pleiomorphic rod-shaped bacterium causing Big Belly disease in fish, characterized in that said bacterium comprises a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ED NO 1 and characterized in that the bacterium comprises a l6 S rRNA gene that reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/-15 base pairs.

In a third aspect, there is provided microbiological culture comprising a bacterium, characterized in that said culture comprises a bacterium as defined above.

In a fourth aspect, there is provided vaccine for combating Big Belly Disease in fish, characterized in that said vaccine comprises a bacterium as defined above and a pharmaceutically acceptable carrier.

In a fifth aspect, there is provided vaccine for combating Big Belly Disease, characterized in that said vaccine comprises antibodies specific to a bacterium as defined above and a pharmaceutically acceptable carrier.

In a sixth aspect, there is provided antibody specific to a bacterium as defined above.

In a seventh aspect, there is provided bacterium as defined above, for use in a vaccine.

In an eighth aspect, there is provided use of a bacterium as defined above, for the manufacture of a vaccine for combating Big Belly Disease.

In a ninth aspect, there is provided method for the preparation of a vaccine as defined above, characterized in that said method comprises the mixing of a bacterium as defined above and a pharmaceutically acceptable carrier.

In a tenth aspect, there is provided diagnostic test kit for the detection of antibodies reactive with a bacterium as defined above, characterised in that said test comprises a bacterium as defined above, or antigenic material thereof.

In an eleventh aspect, there is provided diagnostic test kit for the detection of a bacterium as defined above or antigenic material thereof, characterised in that said test comprises antibodies reactive with a bacterium as defined above.

In a twelve aspect, there is provided diagnostic test kit for the detection of a bacterium as defined above, characterised in that said test comprises PCR-primers that specifically react with the 16 S rRNA gene of a bacterium as defined above.

It has now been determined that the causative agent of this disease is a Gram-negative pleiomorphic rod-shaped bacterium comprising a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1.

SEQ ID NO 1 shows the nucleotide sequence of most of the 16 S rRNA gene of a bacterium according to the invention. With a level of identity is of course meant the level of identity of the sequence of SEQ ID NO 1 and the corresponding region of the 16S rRNA of the bacterium of which the level of identity has to be determined.

It is therefore considered that Gram-negative pleiomorphic rod-shaped bacteria causing Big Belly disease in fish, of which the nucleotide sequence of the region of

the 16 S rRNA gene corresponding to the 16 S rRNA as depicted in SEQ ID NO 1 has a level of identity that is at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % identical to that of the nucleotide sequence as depicted in SEQ ID NO 1, belong to the novel bacterium according to the invention.

Thus, a first embodiment of the invention relates to Gram-negative pleiomorphic rod-shaped bacteria causing Big Belly disease in fish wherein the bacteria comprise a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1.

A typical example of software suitable for the determination of the level of identity is the Vector NTI Advance^{tm} software and settings as described in Example 7.

Organisms sharing more than 97% rRNA identity are considered to belong to a single species (VanDamme et al., Microbiol. Reviews 60: 407-438 (1996)).

Another, alternative, way to characterize the Gram-negative pleiomorphic rod-shaped bacterium according to the invention depends on a PCR-test using primers that are specific for the 16 S rRNA gene sequence of bacteria according to the invention. These primers, of which the sequence is depicted in SEQ ID NO.: 2 and 3, were selected for their specific selectivity for the novel bacterium. They specifically react with the 16 S rRNA gene of the novel bacterium but not with that of closely related bacteria such as *Photobacterium spp.* and *Vibrio spp.* that do not belong to the bacterium according to the invention. The test, which is described in more detail in the Examples section, is a standard PCR test.

It is therefore considered that Gram-negative pleiomorphic rod-shaped bacteria causing Big Belly disease in fish, of which the 16 S rRNA gene reacts in a PCR reaction with primers as depicted in SEQ ID NO.: 2 (BBF-1) and 3 (BBR-4), to give a PCR product of 903 +/- 15 base pairs also belong to the novel bacterium according to the invention.

Thus, the first embodiment also relates to Gram-negative pleiomorphic rod-shaped bacteria causing Big Belly disease in fish, of which the 16 S rRNA gene reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/- 15 base pairs.

A preferred form of this embodiment relates to Gram-negative pleiomorphic rod-shaped bacteria causing Big Belly disease in fish, of which the 16 S rRNA gene has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1 and of which the 16 S rRNA gene reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/- 15 base pairs.

In a more preferred form of this embodiment, the bacterium according to the invention is in an inactivated form, for reasons which will be explained below.

In another more preferred form of this embodiment, the bacterium according to the invention is in a live attenuated form, for reasons which will be explained below.

It is one of the merits of the present invention that the hitherto unknown causative agent of the disease has now been unambiguously determined, confirmed by Koch's postulates.
Now that the cause of the disease has been found and could be demonstrated to be of bacterial origin the disease could deliberately be induced and the typical signs of the disease described hereunder are frequently noticed.
Up till now, the disease is seen in Asian seabass. It is probably only a matter of time before the disease will be demonstrated in other cultured aquatic species.
During a typical outbreak, the mortality lasts for about 1 to 3 weeks in fish from 0.1 g to 5 g. The survival rate is lower than 30% and may even drop to 0% in the worst case. All batches of fish can be affected and the disease appears to occur throughout the year.

Typically, pathogenesis begins with very few small juveniles turning black and thin while the majority of the fish in the tank seem unaffected and eat eagerly. Normally the smallest fish in a batch or tank start displaying the disease signs. However, over a period of 3 to 5 days all fish in the tank develop signs of disease and rapidly die.

Affected fish usually show no external disease signs, no ulcers, no white skin patches, no parasites, no fin or tail rot and no obvious gill necrosis. The first sign is fish turning black instead of keeping their normal brownish color. Subsequently they stop to feed, the tail section becomes very thin. The larvae stop swimming and can be found separated from the group near the bottom of the tank. Subsequently the abdomen swells visibly and fish can be seen as "Big-Belly" fish. Within hours the fish die.

The disease has been designated Big Belly Syndrome (BBS), thus referring to one of the specific signs of the syndrome, the typical big belly.

The following internal pathology is often seen: the internal organs are lumped together in an indistinguishable mass with slight hemorrhages in the liver and some parts of the digestive tract (when distinguishable). The spleen is usually intact and very red; all other organs are engulfed in an apparent mass of fat with yellowish and whitish apparently auto-digested substances.

The newly discovered causative agent of the syndrome is now determined to be a Gram-negative rod-shaped bacterium. The novel bacterium is found both freely in/between the tissues and intracellular inside host cell, apparently in vacuoles. The novel bacterium is, although highly pleiomorphic, generally 2-4 μm long and 1-2 μm wide and often contains an intracellular vacuole that typically stains red in a Giemsa stain.

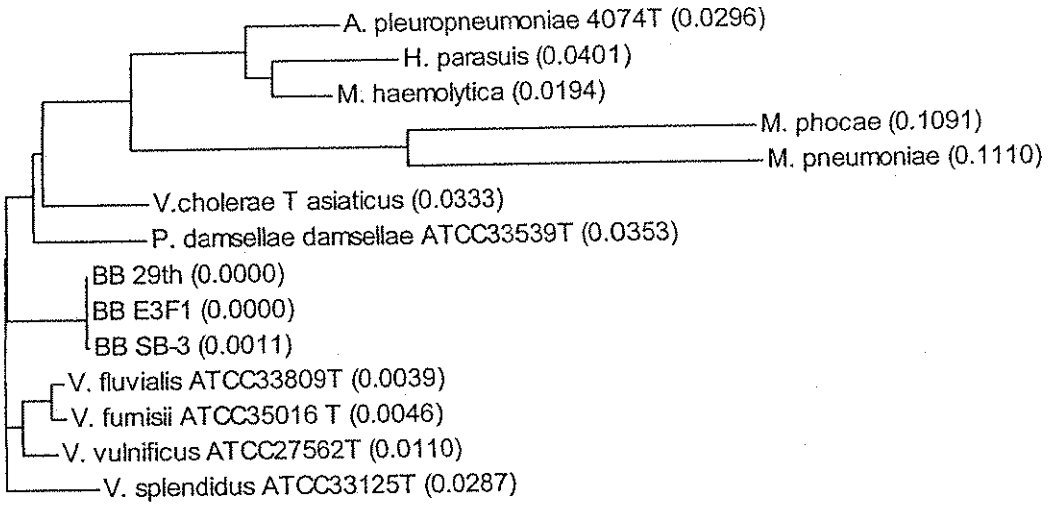
An example of the novel bacteria (BB E3F1) has been deposited with the Collection Nationale de Cultures de Microorganisms (CNCM), Institut Pasteur, 25 Rue du Docteur Roux, F-75724 Paris Cedex 15, France, under accession number CNCM I-3257.

The novel bacterium belongs to a hitherto unknown species. Most of its 16 S ribosomal RNA (rRNA) gene sequence has been determined and the part of that sequence that is depicted in SEQ ID NO 1 reveals a level of identity that is significantly lower than 97% when compared with even the most closely related genera/species: *Vibrio* and *Photobacterium*. Comparison of the currently available 16 S rRNA gene sequences of the various *Photobacterium* species with that of the bacterium according to the present invention reveals a maximum level of identity of 93% (for *P. damselae*). Comparison with the currently available 16 S rRNA gene sequences of the various *Vibrio* species reveals a maximum level of identity of 95% (for *V. fluvialis, V.furnisii* and *V. vulnificus*). Table 1 shows the level of identity of the 16 S rRNA gene of the novel bacterium with various bacterial families/species.

	A. pleuropneumoniae 4074T	H. parasuis	M. haemolytica	M. phocae	M. pneumoniae	V.cholerae T asiaticus	P. damsellae damsellae ATCC33539T	BB 29th	BB E3F1	BB SB-3	V. fluvialis ATCC33809T	V. furnisii ATCC35016T	V. vulnificus ATCC27562T	V. splendidus ATCC33125T
A. pleuropneumoniae 4074T	100	92	94	71	70	87	87	86	86	86	86	86	87	86
H. parasuis		100	94	68	69	85	84	84	84	84	85	85	85	84
M. haemolytica			100	70	71	87	86	87	87	86	86	86	87	86
M. phocae				100	75	71	69	70	70	70	70	70	70	70
M. pneumoniae					100	70	70	70	70	70	70	70	70	70
V.cholerae T asiaticus						100	92	92	92	92	93	93	94	92
P. damsellae damsellae ATCC33539T							100	93	93	93	94	94	93	92
BB 29th								100	100	100	95	95	95	94
BB E3F1									100	100	95	95	95	94
BB SB-3										100	95	95	95	94
V. fluvialis ATCC33809T											100	99	97	95
V. furnisii ATCC35016 T												100	97	95
V. vulnificus ATCC27562T													100	95
V. splendidus ATCC33125T														100

Table 1

As can be seen in the dendrogram below, the novel bacterium according to the invention, the cause of Big Belly Disease as exemplified by three different Big Belly strain isolates; BB 29th, BB E3F1 and BB SB-3, forms a distinguished species, clearly apart from the bacteria of the HAMP-group (*Haemophilus, Actinobacillus, Mannheimia, Pasteurella*), from *Vibrio* and from the *Photobacteriae*.



Basically, a typical PCR-based test applied for the discrimination between the novel bacterium according to the invention and other pathogens could be based upon the following:

The primer set to be used in the test is BBF-1/BBR-4 (see below):

BBF-1	5' TGAGTAATGCCTAGGAACA	Forward
BBR-4	5' TCTCCGCTGGCTTCTGTA	Reversed

A small portion of a colony, but at least 10 cells, preferably 10^3 cell (volume about 1 µl), is to be picked from a suitable agar plate (see Examples) and transferred to a tube containing puReTaq Ready-To-Go PCR beads dissolved in 23 µl of double distilled water (Amersham Biosciences Cat no. 27-9558-01). Subsequently, 0.5 µl of each of the primers BBF-1 with BBR-4 (10 µM stock solution) is added. The sample is subsequently run using a thermal cycler with the following settings:

Denaturation	95°C	2mins	x 1 cycle
Amplification	95°C	30secs	
	56°C	45 secs	x 30 cycles
	72°C	45 secs	
Extension	72°C	5mins	x 1 cycle

If analysis of the PCR-product reveals a PCR product of approximately 903 base pairs, this unequivocally demonstrates that the analysed bacterium belongs to the novel bacterium according to the invention. A PCR product of approximately 903 base pairs is a PCR product with a length of between 888 and 918 base pairs.

As will be discussed *in extenso* in the Examples below, another of the merits of the present invention is, that a suitable growth medium for the bacterium according to the invention has now been found. Bacteria according to the invention can now be grown in vitro.

The skilled person finds in Example 3 below a method for the isolation of the bacterium according to the invention from diseased fish, as well as for further growth of the bacterium on suitable medium.

Therefore, another embodiment of the present invention relates to a microbial culture comprising a bacterium according to the invention.

In a preferred form of this embodiment, the microbial culture comprises NAD.

It is again one of the merits of the present invention that, the causative agent being known now, the development of vaccines became feasible.
The strong immune response triggered in infected fish, which leads to the induction of immunity against a second infection with the bacterium according to the invention is in itself already an indication that vaccination is feasible. The problem encountered

in the natural course of the disease however is, that the onset of an adequate immune response usually is too slow. An adequate immune response, i.e. a response that suppresses the infection to at least a level that enables the fish to survive the infection, takes time to build up. Under natural conditions, this time is usually not available due to the very rapid progress of the disease: 90% of the infected fish die within days. Because the pathogen causing the disease has now been identified, a vaccine based upon this pathogen would solve this problem because after vaccination, the immunological defense against the bacterium can build up before a natural infection strikes.

Thus, another embodiment of the present invention relates to a vaccine for combating the disease; Big Belly Disease, as caused by the novel bacterium, wherein said vaccine comprises a bacterium according to the invention and a pharmaceutically acceptable carrier.

The vaccine according to the invention may comprise the bacteria in attenuated live or inactivated form.
Attenuated live vaccines, i.e. vaccines comprising the bacterium according to the invention in a live attenuated form, have the advantage over inactivated vaccines that they best mimic the natural way of infection. In addition, their replicating abilities allow vaccination with low amounts of bacteria; their number will automatically increase until it reaches the trigger level of the immune system. From that moment on, the immune system will be triggered and will finally eliminate the bacteria.
A minor disadvantage of the use of live attenuated bacteria however might be that inherently there is a certain level of virulence left. This need not be a real disadvantage as long as the level of virulence is acceptable, i.e. as long as the vaccine at least prevents the fish from dying. Of course, the lower the rest virulence of the live attenuated vaccine is, the less influence the vaccination has on weight gain during/after vaccination.

Therefore, one preferred form of this embodiment of the invention relates to a vaccine comprising a bacterium according to the invention in a live attenuated form.

A live attenuated bacterium is a bacterium that has a decreased level of virulence when compared to field strains. As mentioned above, the virulence of field strains of the novel bacterium according to the invention is very high: mortality typically exceeds 70% of all infected fish. A bacterium having a decreased level of virulence is considered a bacterium that only induces disease to the extent that mortality does not exceed 10%, and 90 % of all infected fish survive the infection. Bacteria often behave attenuated as a result of a decreased growth rate. If such bacteria are used as the basis of an attenuated live vaccine, contrary to the situation described earlier, the immune system is triggered to the level necessary to suppress the disease before the fish die. As a result, the fish will not only survive but additionally, they build up immunity against future infections with a fully virulent field strain.
Attenuated strains can e.g. be obtained by growing the bacteria according to the invention in the presence of a mutagenic agent. Many of such agents are known in the art. Another way of obtaining mutated bacteria is to subject them to growth under temperatures exceeding the temperature of their natural habitat. Yet another way of mutating bacteria well-known in the art is transposon-mutagenesis.

Selection methods for slow-growing mutants or for temperature sensitive mutants (Ts-mutants) are well-known in the art. Merely as an example: a suitable method for selection of slow-growing mutants is simply plating of bacteria treated with a mutagen followed, after incubation, by visual selection of small colonies. Such colonies are slow-growing and thus desired live attenuated bacteria.
Selection for Ts-mutants is equally easy: replica-plating of bacteria treated with a mutagen followed by incubation at a sub-optimal (2-4 degrees below native growth temperature) or the optimal temperature, followed by visual selection of those colonies that did grow normal speed under sub-optimal temperature but did grow slower at the optimal temperature.

Inactivated vaccines are, in contrast to their live attenuated counterparts, inherently safe, because there is no rest virulence left. In spite of the fact that they usually comprise a somewhat higher dose of bacteria compared to live attenuated vaccines,

they may e.g. be the preferred form of vaccine in fish that are suffering already from other diseases. Fish that are kept under sub-optimal conditions, such as incomplete nutrition or sub-optimal temperatures, would also benefit from inactivated vaccines.

Therefore, another preferred form of this embodiment relates to a vaccine comprising a bacterium according to the invention in an inactivated form.

Many physical and chemical methods of inactivation of bacteria are nowadays known in the art. Examples of physical inactivation are UV-radiation, X-ray radiation, gamma-radiation and heating. Examples of inactivating chemicals are ß-propiolactone, glutaraldehyde, ethylene-imine and formaldehyde. The skilled person would undoubtedly know how to apply these methods.
Preferably the strain is inactivated with ß-propiolactone, glutaraldehyde, ethylene-imine or formaldehyde. It is obvious that other ways of inactivating the bacteria are also embodied in the present invention.

Vaccines comprise the bacterium according to the invention in an attenuated live and/or killed form, and in addition they comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be as simple as e.g. distilled water or a physiological salt solution. It can also be e.g. a buffer solution.

Vaccines according to the present invention may in a preferred presentation also contain an immunostimulatory substance, a so-called adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants frequently used in fish and shellfish farming are muramyldipeptides, lipopolysaccharides, several glucans and glycans and Carbopol(R) (a homopolymer). An extensive overview of adjuvants suitable for fish and shellfish vaccines is given in the review paper by Jan Raa (Reviews in Fisheries Science 4(3): 229-288 (1996)).
The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the bacterium adheres, without being covalently bound to it. Such vehicles are

i.a. bio-microcapsules, micro-alginates, liposomes and macrosols, all known in the art.

A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span or Tween.

Oil adjuvants suitable for use in water-in-oil emulsions are e.g. mineral oils or metabolisable oils. Mineral oils are e.g. Bayol®, Marcol® and Drakeol®.

Metabolisable oils are e.g. vegetable oils, such as peanut oil and soybean oil, animal oils such as the fish oils squalane and squalene, and tocopherol and its derivatives.

Suitable adjuvants are e.g. w/o emulsions, o/w emulsions and w/o/w double-emulsions

Very suitable o/w emulsions are e.g. obtained starting from 5-50% w/w water phase and 95-50% w/w oil adjuvant, more preferably 20-50% w/w water phase and 80-50% w/w oil adjuvant.

The amount of adjuvant added depends on the nature of the adjuvant itself, and information with respect to such amounts will be provided by the manufacturer.

Often, the vaccine is mixed with stabilisers, e.g. to protect the bacteria from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

Preferably, vaccines according to the invention are stored/presented in a freeze-dried form.

In addition, the vaccines may be suspended in a physiologically acceptable diluent.

It goes without saying, that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilising are also embodied in the present invention.

Vaccines based upon inactivated bacteria can in principle be administered in doses between 10^3 and 10^9 CFU bacteria. Doses below 10^3 bacteria might, depending i.a. on the method of inactivation, not be sufficiently immunogenic, and doses that exceed 10^9 bacteria would from a commercial point of view not be very attractive.

Vaccines based upon live attenuated bacteria can in principle be administered in lower doses, because they multiply themselves during the infection. Therefore, although suitable amounts would range between 10^3 and 10^9 CFU bacteria, amounts between 10^3 and 10^6 CFU are preferred amounts.

Many ways of administration, all known in the art can be applied. The vaccines according to the invention are preferably administered to the fish via injection, immersion, dipping or per oral. The administration protocol can be optimized in accordance with standard vaccination practice. Preferably the vaccine is administered via immersion or per oral, especially in case of the use of vaccines in commercial aquaculture farms.

For oral administration the vaccine is preferably mixed with a suitable carrier for oral application i.e. cellulose, food or a metabolisable substance such as alpha-cellulose or different oils of vegetable or animals origin. Also an attractive way is administration of the vaccine to high concentrations of live-feed organisms, followed by feeding the live-feed organisms to the target animal, e.g. the fish. Particularly preferred food carriers for oral delivery of the vaccine according to the invention are live-feed organisms which are able to encapsulate the vaccine.

Suitable live-feed organisms include plankton-like non-selective filter feeders preferably members of Rotifera, *Artemia*, and the like. Highly preferred is the brine shrimp *Artemia* sp..

The administration protocol can be optimized in accordance with standard vaccination practice. A recent overview of fish vaccination, written by Bowden et al., (Fisheries Research Service Marine Laboratory, Aberdeen, Scotland) is available from www.intrafish.com.

The age of the fish to be vaccinated is not critical, although clearly one would want to vaccinate against Big Belly Disease in an early stage. In principle, it would be tempting to vaccinate fish preferably at 0.2 grams, but certainly before 5 grams of weight. Fish having a weight of < 0.5 grams however are assumed to be insufficiently immune competent. Therefore, in practice, one would try to vaccinate fish having a weight of between 0.5 and 5 grams. Since it is one of the merits of the present invention that it is now possible to perform early diagnosis of the bacterium and of the disease, control measurements such as sanitation can be developed in order to postpone outbreaks until fish have been vaccinated.

It would be beneficial to add to a vaccine, together with bacteria according to the invention, also at least one other fish-pathogenic microorganism or virus, an antigen of such microorganism or virus or genetic material encoding such an antigen in a combination-vaccine.

Examples of notorious commercially important fish pathogens are *Vibrio anguillarum, Photobacterium damselae* subspecies *piscicidae, Tenacibaculum maritimum, Flavobacterium sp., Flexibacter sp., Streptococcus sp., Lactococcus garviae, Edwardsiella tarda, E. ictaluri,* Viral Necrosis virus, iridovirus and Koi Herpesvirus.

The advantage of such a combination vaccine is that it not only provides protection against Big Belly Disease, but also against other diseases.

Therefore, a preferred form of this embodiment relates to a vaccine wherein that vaccine comprises at least one other microorganism or virus that is pathogenic to fish, or one other antigen or genetic material encoding said other antigen, wherein said other antigen or genetic material is derived from a virus or microorganism pathogenic to fish.

Thus, in a more preferred form of this embodiment, the other microorganism or virus is selected from the following group of notorious commercially important fish pathogens: *Vibrio anguillarum, Photobacterium damselae* subspecies *piscicidae, Tenacibaculum maritimum, Flavobacterium sp., Flexibacter sp., Streptococcus sp., Lactococcus garviae, Edwardsiella tarda, E. ictaluri,* Viral Necrosis virus, iridovirus and Koi Herpesvirus.

All vaccines described above contribute to active vaccination, i.e. they trigger the host's defense system.

Alternatively, antibodies can be raised against the bacterium according to the invention in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the fish. This method of vaccination, passive vaccination, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for vaccinating animals that are prone to sudden high infection pressure. The administered antibodies reactive with the bacterium according to the invention can in these cases interfere with the bacterium according to the invention and thus suppress Big Belly Disease. This approach has the advantage that it decreases or stops Big Belly Disease development, independent of the fish' immune status.

Therefore, one other form of this embodiment of the invention relates to a vaccine for combating Big Belly Disease that comprises antibodies reactive with bacteria according to the invention and a pharmaceutically acceptable carrier.

Still another embodiment of this invention relates to antibodies reactive with bacteria according to the invention.

Antibodies or antiserum against bacteria according to the invention can be obtained quickly and easily by vaccination of e.g. pigs, poultry or e.g. rabbits with inactivated bacteria according to the invention in e.g. a water-in-oil suspension followed, after

about four weeks, by bleeding, centrifugation of the coagulated blood and decanting of the sera.

Another source of antibodies is the blood or serum of e.g. seabass that have been naturally infected with bacteria according to the invention.
Other methods for the preparation of antibodies, which may be polyclonal, monospecific or monoclonal (or derivatives thereof) are well-known in the art. If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are well-known in the art (e.g. Mayer and Walter, eds. Immunochemical Methods in Cell and Molecular Biology, Academic Press, London, 1987). Monoclonal antibodies, reactive against the novel bacterium according to the invention can be prepared by immunizing inbred mice by techniques also known in the art (Kohler and Milstein, Nature, 256, 495-497, 1975).

A vaccine can also be prepared using antibodies prepared from eggs of chickens that have been vaccinated with a vaccine according to the invention (IgY antibodies).

Preferably a vaccine for oral administration of the antibodies is prepared, in which the antibodies are mixed with an edible carrier such as fish food.

Still another embodiment relates to a method for the preparation of a vaccine according to the invention that comprises the admixing of a bacterium according to the invention and a pharmaceutically acceptable carrier.

Still another embodiment relates to a method for the preparation of a vaccine according to the invention that comprises the admixing of antibodies reactive with a bacterium according to the invention and a pharmaceutically acceptable carrier.

Again another embodiment of the present invention relates to bacteria according to the invention for use in a vaccine.

Still another embodiment of the present invention relates to the use of a bacterium according to the invention for the manufacture of a vaccine for combating Big Belly Disease.

As mentioned above, lethality after bacterial infection can easily be up to 70-90% and can even reach 100%. In addition to this, disease strikes at a dramatically high speed. Thus, for efficient protection against disease, a quick and correct diagnosis of Big Belly Disease is important.

Therefore it is another objective of this invention to provide diagnostic tools suitable for the detection of Big Belly Disease.

A diagnostic test kit based upon the detection of a bacterium according to the invention or antigenic material of that bacterium and therefore suitable for the detection of bacterial infection may i.a. comprise a standard ELISA test. In one example of such a test the walls of the wells of an ELISA plate are coated with antibodies directed against the bacterium. After incubation with the material to be tested, labeled antibodies reactive with the bacterium are added to the wells. A color reaction then reveals the presence of antigenic material of the bacterium.
Therefore, still another embodiment of the present invention relates to diagnostic test kits for the detection of antigenic material of the bacterium. Such test kits comprise antibodies reactive with a bacterium according to the invention or antigenic material thereof. Antigenic material of the bacterium is to be interpreted in a broad sense. It can be e.g. the bacterium in a disintegrated form, or bacterial envelope material comprising bacterial outer membrane proteins, just to name a few. As long as the material of the bacterium reacts with antiserum raised against the bacterium, the material is considered to be antigenic material.

A diagnostic test kit based upon the detection in serum of antibodies reactive with the bacterium according to the invention and therefore suitable for the detection of Big Belly Disease may also i.a. comprise a standard ELISA test. In such a test the walls of the wells of an ELISA plate can e.g. be coated with the bacterium according to the

invention or antigenic material thereof. After incubation with the material to be tested, labeled antibodies reactive with the bacterium according to the invention are added to the wells. A lack of color reaction then reveals the presence of antibodies reactive with he bacterium according to the invention.

Therefore, still another embodiment of the present invention relates to diagnostic test kits for the detection of antibodies reactive with the bacterium. Such test kits comprise the bacterium according to the invention, or antigenic material thereof.

The design of the immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labeled antibodies; the labels may be, for example, enzymes, fluorescent-, chemoluminescent-, radio-active- or dye molecules.

Suitable methods for the detection of antibodies reactive with a bacterium according to the present invention in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescense test (IFT) and Western blot analysis.

A very quick and easy diagnostic test for diagnosing the presence or absence of a bacterium according to the invention is a PCR test as described above, comprising PCR-primers BBF-1 and any of the primers BBR-2, -3 or -4. Preferably, such a test would use the primer combination BBF-1/BBR-4 as depicted in SEQ ID NO.: 2 or 3.

It goes without saying, that more primers can be used than the four primers identified above. The present invention provides for the first time the unique sequence of the 16 S rRNA gene of Big Belly strains. This allows the skilled person to select without any additional efforts, other selective primers by simply repeating the method described in Example 7 for the selection of the four primers shown there. By simple computer-analysis of the 16 S rRNA gene sequence provided by the present invention with the, known, 16 S rRNA gene sequence of other bacteria, the skilled person is able to develop other specific PCR-primers for diagnostic tests for the detection of a Big

Belly strain and/or the discrimination between Big Belly strain and other bacterial (fish) pathogens.

PCR-primers that specifically react with the 16 S rRNA gene of Big Belly strains are understood to be those primers that react only with the 16 S rRNA gene of Big Belly strains and not with the 16 S rRNA gene of another (fish) pathogenic bacterium, or group of (fish) pathogenic bacteria.

Thus, another embodiment relates to a diagnostic test kit for the detection of a bacterium according to the invention, which test has as a characteristic feature that it comprises PCR-primers that specifically react with the 16 S rRNA gene of Big Belly strains.

A preferred form of this embodiment relates to the specific PCR-primers BBF-1 and BBR-2, -3 or -4.

A more preferred form of this embodiment relates to such specific PCR-primers BBF-1 and BBR-4, as depicted in SEQ ID NO.: 2 or 3.

EXAMPLES

Example 1: Primary isolation of the organisms and establishment of a primary isolation medium.

In order to check if the causative agent of the disease could be a bacterium and, if so, in order to be able to isolate the hitherto unknown pathogen, a small batch (200 fish) of fish showing disease signs was brought over to the lab.

Direct wet mounts and impression prints clearly showed the presence of bacteria.

Samples of the internal organs were plated onto a variety of media, namely:

Blood agar (TSA + 5% horse blood – BioMerieux)
Trypticase Soy Agar with 1% NaCl
Trypticase Soy Agar without 1% NaCl
Luminescence medium with 0.5% NaCl
Brain Heart Infusion agar

Thiosulphate Citrate Bile salts Sucrose agar
Vitox medium
Marine agar
MacConkey agar
Arnacker and Ordal medium
Arnacker and Ordal medium prepared with seawater
Caseinase agar
Streptococcus growth medium

On none of these media a growth level was obtained which corresponded to the abundance of the bacteria seen in the impression prints.

However, from 3 fish, small transparent colonies were found in the direct vicinity of Gram-positive contaminants, i.e. bacterial colonies occurring on the plates as a contamination after 3-5 days incubation at 26°C. Only few of these small transparent colonies appeared and then only located closely to the contaminants. Gram-staining of these colonies revealed very strangely formed bacteria of variable sizes and often approaching eukaryotic cell dimensions. These colonies were only found on blood agar plates and not on any other medium. Blood agar plates without contaminants did not show these colonies.

Subcultures of these were tried on Blood Agar and on Blood agar with the contaminants, the latter in a 'cross-feeding" inoculation. Subcultures were found to be present only when the cross-feeding strain was present. Furthermore, growth only occurred when a rather large amount of inoculum was used and growth only started at the first streak and than progressed slowly towards subsequent streaks. The data illustrated that the novel bacteria required nutrients or co-factors which they cannot produce themselves from other bacteria.

Since co-factors were apparently required, it was then evaluated whether the bacteria could grow on:

Marine agar with fresh yeast extract

Marine agar with yeast autolysate (Oxoid)

Marine agar with Vitox (Oxoid)

However, none of these enriched media showed growth.

The ability to grow in absence of presence of CO_2 (5%) was evaluated with and without cross feeding. Only cross fed-colonies grew and CO_2 did not increase the growth performance.

Subsequently it was evaluated whether the strains could be grown in cell culture, given the apparent cell-bound nature as was found in impression prints. Growth was evaluated in Brown Bullhead cells and in grass Carp Ovary cells both in and without the presence of antibiotics. None of the cell cultures yielded growth.

In further tests it was found that the strains could however be subcultured, without cross-feeder strains, on Mycoplasma medium (Mycoplasma agar base; NAD, L-Cysteine-HCl and Fresh Yeast Extract).

Additional testing showed that the bacteria grew very good on Chocolate Agar supplemented with Vitox (Oxoid). Vitox contains NAD (V-factor), Vitamin B12, Thiamine hydrochloride and various minerals and growth factors.
Also suitable, instead of Vitox, is IsoVitalex (BBL).

The combinations of the above isolation data suggest that the media for isolation require the addition of NAD as a factor for growth. This is also in line with the initial observation that cross-feeding was required on normal media. (At the moment, mutated strains independent of these co-factors have also been found).

Chocolate agar supplemented with Vitox turned out to be the best medium (of the media tested so far) for growth. Culture condition evaluation indicates that plates can be incubated at normal atmosphere at 26°C.

Example 2: development of Big Belly Disease.

In order to study the disease, a batch of juvenile seabass without any disease signs was placed in a clean water recirculation system, equipped with an in-line UV system. Contamination of healthy fish through polluted water could therefore be excluded.

However, disease and mortality also developed under these conditions, indicating that the disease was already present in (some of) the fish. Two days after arrival, some fish became dark and thin, swimming out of the group. Subsequently these fish developed Big Belly Disease and died. Over the next days mortality increased dramatically leaving 10% live fish at the end of the mortality period. The fish that survived remained unaffected.

The mortality as observed in this batch of fish is given in Figure 1.

This experiment has provided insight in the course of the disease after infection.

Example 3: confirmation of suitability of the primary isolation medium as a medium for direct isolation of the bacterium according to the invention from diseased fish.

In order to confirm the suitability of Chocolate agar supplemented with Vitox as primary isolation medium we sampled the internal organs of diseased fish (in particular the fat/granulomatous tissue in the abdomen) directly onto Chocolate agar supplemented with Vitox. Two days after inoculation clear and massive growth was obtained from all fish sampled. The direct growth of bacteria from these samples shows and confirms that direct and massive isolation of the organism was possible and this confirmed the likelihood that the organism isolated was identical to the organism observed in Giemsa stained impression prints.

Subsequently, from another Seabass hatchery in Singapore (unrelated to the farm in Indonesia) juveniles with similar disease signs were sampled. Direct impression prints from these fish as well as the macroscopic evaluation of disease signs and internal organs showed the presence of similar organisms. Samples from these fish were plated as described above for the Indonesian samples. All samples plated yielded massive growth of one single colony type, similar to the earlier found colonies.

This implies that for the skilled person looking for an example of the bacterium according to the invention, it suffices to obtain a fish suffering from Big Belly disease and sample the internal organs directly onto chocolate agar supplemented with Vitox as primary isolation medium for the growth of the bacterium.

An overview of strains obtained from different origins is given in Table 2.

Table 2: Strain collection of Big Belly bacteria

Strain code	Isolation origin	Country of isolation	Original code	Isolation medium
INS 445	seabass	Indonesia	10^{th} P	BA
INS 454	seabass	Indonesia	SB-3	BA
INS 512	seabass	Indonesia	E3F1	Mycoplasma
INS 527	seabass	Indonesia	F4	BA
INS 528	seabass	Indonesia	F3	BA
INS 529	seabass	Indonesia	F1	BA
SB 1127	seabass	Indonesia	BB-3	Choco +
SB 1128	seabass	Indonesia	BB-4	Choco +
SB 1129	seabass	Indonesia	BB-5	Choco +
SB 1138	seabass	Indonesia	BB-2	Choco +
INS 598	seabass	Singapore	F1	Choco +
INS 599	seabass	Singapore	F2	Choco +
INS 600	seabass	Singapore	F3	Choco +
INS 601	seabass	Singapore	M9	Choco +

Example 4: Identification and histology

Light microscopy.

All strains are Gram negative and very pleiomorphic in appearance. Some bacteria showed an intracellular black dot (vacuole). Colonies did not swarm and showed a typically raised, white center. They had a light translucent outer rim and were non pigmented. They were non-haemolytic when tested on horse blood agar.

Fresh tissues from fish were pressed lightly on clean glass slides, air dried, and stained with either Giemsa stain or Gram stain according to standard procedures.

First observations of the presence of an infective organism were made through impression prints from internal organs of affected fish. Typically, the internal organs of affected fish were linked together as a mass, comparable to massive adhesions and an apparent proliferation of fat tissue.

In impression prints groups of organism were seen of 1 to 5 μm long, located between host cells. Typically the organisms were clustered and appeared as if they are located inside cells.

In Gram-staining the organism stained Gram-negative and often showed a clear halo around the stained cell, indicating the presence of capsular material.

In Giemsa-staining more details of the organism became apparent. Typically the organisms displayed bipolar staining (a trait often observed and described for the genus *Pasteurella*) and often displayed a bright red to orange intracellular spot or vacuole. Figure 2 is a Giemsa-stained impression print showing the above indicated characteristics. This shows a Giemsa-stained impression print of the spleen of Asian seabass showing bipolar appearance (double arrow) and the "vacuole" (single arrow).

Conclusion

Coccoid to rod-shaped, basophilic, Gram-negative, intracellular proliferating microorganisms were observed. A severe necrosis followed by severe granulomatous reaction could be observed in internal abdominal organs and tissues.

A severe, chronic, granulomatous, necrotic inflammatory reaction was observed in the abdominal organs. The main affected tissues were intestine (posterior from stomach) and visceral fat tissue.

Electron microscopy

Tissues were fixed in Glutaraldehyde and processed to ultra-thin sections according to standard electron microscopical methods.

EM analysis of the sections showed numerous, relatively large (2 by 1 μm), ellipsoid organisms. These organisms are surrounded by a membrane or cell wall. They could

be found either free in the tissues or intracellular, surrounded by a host cell membrane, i.e. in a vacuole. Characteristically, the organisms contain an inclusion body or vacuole (300 nm diameter) containing non-electrondense material.

Figure 3 shows an EM picture of the intracellular organisms.

Based on the morphological appearance it was concluded that the organisms were most likely rather large bacteria.
Further analysis showed that the organisms were surrounded by a biological double membrane (not shown) and that they did not posses a nucleus. These observations rule out the possibility of a protozoan and are in line with Gram-negative bacteria. In conclusion, the organisms are Gram negative bacteria.

Conclusion and discussion

Based on the overall histological analysis it is concluded that the organisms observed in Asian seabass (*Lates calcarifer*) suffering from BBS are Gram-negative bacteria. The bacteria are found both free in the tissues and intracellular inside host cell vacuoles. The bacteria are generally 2 µm long and 1 µm wide and often contain an intracellular vacuole that typically stains red in a Giemsa stain.

Example 5: serology

Agglutination.

Blood and subsequent serum was collected from surviving fish from an outbreak (The case shown in Figure 1). This serum was subsequently used in slide agglutination tests to evaluate the serotype of the isolates given in Table 2.

All strains agglutinated with the antiserum, whereas they did not with seabass serum collected from fish that didn't went through a BBS-outbreak (collected in 2000). These results indicate that all strains of BBS bacteria belong to the same serotype.

ELISA.

Six strains, representing different time-points of isolation as well as the two countries where the novel bacterium according to the invention was isolated, were further tested in an ELISA. The strains used are listed in Table 3. Bacterial strains were grown for 48 hours at 26°C on Chocolate agar supplemented with Vitox and harvested in Sodium-Acetate buffered Formalin saline (Grisez, L. and F. Ollevier, Appl. Environm. Microbiol, 61:12; 4367-4373 (1995)). The resulting suspension was kept overnight at 4°C and subsequently centrifuged at 14,000 RPM and the supernatant was discarded. The cells were re-suspended in 40 mM PBS (pH 7.2) and brought to an optical density of approximately 0.250 at 660 nm wavelength. From these standardized bacterial suspensions 100 µl per well were then coated over two rows per strain onto 96-well ELISA plates (Nunc Maxisorp) and incubated for 2 hours at 26°C followed by an overnight incubation at 4°C.

Table 3: Strains used for titer determination through ELISA

Strain code	Isolation origin	Country of isolation
BB 445	seabass	Indonesia
BB 454	seabass	Indonesia
BB 512	seabass	Indonesia
BB 527	seabass	Indonesia
BB 1128	seabass	Indonesia
BB 598	seabass	Singapore

Table 3.

After removal of the buffer, blocking the non-specific binding sites with a 3% (w/v) skim milk solution and washing, the primary antiserum was added. One row per strain was incubated with 100 µl positive seabass serum, i.e. the serum collected from survivors of a recent natural infection, whereas the second row per strain was incubated with negative seabass serum. Both sera were pre-diluted 16 times before adding them to the first column and further serially two-fold diluted from column 1 to column 10. Columns 11 and 12 served as negative controls in which the primary antiserum was not included for column 11 and the secondary antibody was not included for column 12. The plates were then incubated for 1 hour at 37°C, washed and further developed using a monoclonal antiserum against European seabass IgM (WDI 1) at a dilution 1:50 and horse radish peroxidase conjugated goat anti mouse (dilution 1:2,000). Reactions were developed by the addition of 3, 3', 5, 5'-tetramethylbenzidine as substrate and read at 450 nm after stopping the reaction by the addition of 50 µl of a 2 M H_2SO_4 solution.

Titers for the different strains were expressed as the reciprocal of the serum dilution given an OD_{450nm} reading of 0.4 after subtracting the negative serum value. The titers obtained for the 6 strains tested are given in Figure 4.

As is shown in figure 4, all strains reacted similarly with the antiserum and a fairly high specific antibody titer was present in the serum. The lowest titer obtained was

372 whereas the highest titer was 1965. No systematic difference was observed between isolation time and geographical place from where the strains were isolated.

It can therefore be concluded that all strains tested share common antigens which are recognized by fish. Furthermore, fish that underwent an infection and survived this infection mount an antibody reaction which plays a key role in clearing the pathogen and inducing subsequent immunity for the pathogen.

Example 6:Koch's postulates

In order to make sure that the assumed pathogen is indeed a pathogen, Koch's postulates have to be fulfilled. In order to evaluate Koch's postulates, an attempt was made to perform a challenge with a bacterium according to the invention.

Materials and methods

Bacterial culture.

The Big Belly bacterium was subcultured by spread plating onto BA (20 plates) and incubated at 26°C for 48 hours. Subsequently the bacterial growth from all plates was combined and subsequently resuspended in 8 ml buffer.

Fish

A total of 40 early juvenile fish Asian seabass were obtained from a hatchery where no BBS was found previously. A number of fish from the same batch were tested by means of Giemsa-staining the internal organs to confirm the absence of the pathogen. All fish analyzed were shown to be free of the pathogen.

The fish had a mean weight of 0.4 g at the moment of challenge with a mean total length of 3.3 cm.

The fish were divided as they came at hand in two equally sized groups of 20 fish and transferred to a 50 L aquarium equipped with a filter.

Challenge

The 8 ml of bacterial suspension was mixed with 792 ml of seawater and 1 group of fish (20 fish) was immersed in the suspension for 30 minutes. Water temperature during challenge was maintained at 28°C and dissolved oxygen was kept above 4 mg/L by means of supplying compressed air.

Observations after challenge.

Dead fish were collected twice daily from the tanks and recorded on record sheets. From all dead fish, a piece of the internal organs was taken (usually a part of the abdominal body fat) and impression prints were made and Giemsa-stained. Observations of the slides with light microscopy at 100x magnification easily

revealed the presence or absence of the Big Belly bacterium. Additionally, the internal organs of a representative number of fish from both groups were plated onto both BA and Mycoplasma agar supplemented with NAD, L-cysteine and Fresh Yeast Extract, irrespective of the results of the Giemsa staining. Plates were incubated at 26°C for 48 hours and the presence of Big Belly bacterial colonies was observed and subsequently confirmed by Gram-staining.

Results

Fish showing the typical disease signs, i.e. distended abdomen, darkened fish swimming out of the group and behaving lethargically, were seen in the challenged group but not in the control group from day 5 after challenge onwards.

Mortality started on day 6 after challenge in the challenged fish and continued over the following 5 days after which all but one fish were dead. The one remaining fish in the challenged group died on day 19 after challenge. All dead fish were sampled and analyzed and the typical bacterium according to the invention was clearly observed in Giemsa stained impression prints.

Fish that died from the control group during the observation period never showed any disease signs and never displayed the typical BBS-bacteria in Giemsa stained impression prints of the internal organs.

Irrespective of the results from the Giemsa-staining, the internal organs (mostly the fat tissue) of a representative number of fish from both groups was plated onto a suitable medium. The novel bacterium according to the invention was successfully re-isolated from 13 sampled challenged fish, but could not be isolated from 3 control fish that had died (mortality had occurred on day 4 post challenge). In addition, all remaining fish in the control group were sectioned at the end of the 21 day observation period, but in none of the fish could the Big Belly bacterium be demonstrated by Giemsa staining or by plating.

The mortality in function of time after challenge, confirmed to be attributed to the challenge organism by either Giemsa staining and/or plating from both the challenged group and the control group is presented in Figure 5.

Discussion

Koch's postulates are defined as:

1) The same organism must be isolated/demonstrated in all cases showing the same disease signs.
2) The suspected pathogenic organism must be isolated as pure and dominant colony type from the animals showing the disease signs.
3) When the suspected pathogenic organism is administered to healthy susceptible animals they should develop the same disease while showing the typical signs of the disease.
4) The same pathogenic organism must again be re-isolated from the challenged animals as a pure and dominant colony type.

The same organism was found in all cases of the disease. All fish showed the same disease signs, i.e. typically a swollen abdomen, internally characterized by severe adhesion of the internal organs into one confluent mass. The same organism was demonstrated and can be recognized and distinguished from other known pathogens by its appearance: mainly intra-cellular, and by its typical morphology: large ellipsoid bacteria (2 x 1 μm), showing bipolar staining in Giemsa stained impression prints together with a orange-red vacuole. Bacteria grown on agar show similar characteristics in Giemsa staining but display very pleiomorphic morphologies in Gram staining.

By using appropriate media, i.e. Chocolate agar supplemented with Vitox, the causative organism could repeatedly be isolated as pure and dominant growth from fish showing the typical disease signs as well as displaying the Big Belly bacterium in Giemsa stained impression prints. By using the correct isolation medium all fish sampled with BBS disease signs yielded the same pathogen. Moreover, the pathogen

was isolated from two distinct and unrelated hatcheries and as such from two different countries (Indonesia and Singapore).

Bacteria isolated from affected fish were used in a challenge experiment (immersion challenge). Healthy Asian seabass juveniles were exposed to a suspension of the challenge organism and all fish exposed to the challenge organism developed the typical BBS disease signs as well as displayed the presence of the challenge organism in the abdominal organs in an appearance identical to what is found in natural outbreaks.

Moreover the challenge organism could be re-isolated from challenged fish as pure and dominant colony type.
Thus, Koch's Postulates are fulfilled, and consequently the relation between BBS and the novel bacterium according to the invention as the causative agent has been proven.

Example 7: Determination of 16 S rRNA sequence and development of a PCR detection method.

Determination of the Big Belly bacterium 16 S rRNA sequence.
Three examples of Big Belly strains were grown on Chocolate agar + Vitox, and harvested. After boiling for 10 minutes, internal fragments of the *rrn* gene encoding the 16S rRNA were subsequently amplified using universal primers and the gene was sequenced using internal primers, essentially as described by Kuhnert et al., Int. J. Syst. Bacteriol 46: 1174-1176 (1996). Basically, the 16S rRNA gene was amplified using oligo's nr 267 (5'-AGAGTTTGATCATGGCTCAG) and nr 268 (5'-GTGTGACGGGCGGTGTGTAC) in a PCR cycling program of 3 minutes denaturation at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 68°C. The program was ended by 7 minutes at 68°C. The amplicons were purified by a Qia-Quick column (Qiagen Inc., California, USA) and individual sequence reactions were performed using the primers mentioned above as well as nr 381 (5'-CTACGGGAGGCAGCAGTGGGG), 382 (5'-

GTGTAGCGGTGAAATGCGTAG). 383 (5'-GGTTAAGTCCCGCAACGAGCGC), 384 (5'-CCCCACTGCTGCCTCCCGTAG), 385 (5'- CTACGCATTTCACCGCTACAC) and 386 (5'-GCGCTCGTTGCGGGACTTAACC). DNA was sequenced on an ABI 310 automated sequencer (Perkin Elmer, California, USA). The sequence mixture contained: 100 ng PCR product, 2 µl terminator ready reaction mix (Perkin Elmer, California, USA), 2.4 pmoles of primer, 6 µl of buffer (200 mM Tris-HCl, pH8.5; 5 mM $MgCl_2$) and aqua dest up to 20 µl. This mixture was cycled on a Geneamp 9700. The program was composed of 25 cycles with 10 seconds 96°C, 5 seconds 50°C and 2 minutes 60°C. The cycle sequence products were purified with Dye-Ex columns (Qiagen Inc., California, USA) according to manufacturers instructions and run on the ABI 310 automated sequencer. Sequence results were collected using ABI 310 Collection Software version 1.0.4 (Perkin Elmer, California, USA) and analysed with Sequence Analysis version 3.1 (Perkin Elmer, California, USA). Alignments were made using Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, USA).

The obtained sequences (1411 nucleotides each) were compared with each other and with published sequences in the public databases on the Interblast server (http://shmsgi01.shm.intra/bci/interblast.html) using BLASTN (see highest scores in table 1). The highest scoring organisms were all in the Vibrio genus and some in the Photobacterium genus. Subsequently, the *rrn* sequences of these genera were downloaded from the Ribosomal RNA Database site (http://rdp.cme.msu.edu/cgis/) and aligned using Vector Advance software (Vector NTI Advance™ software, as available from www.Invitrogen.com). Pairwise and multiple alignment settings: open gap penalty 15; gap extension penalty 6.66). The resulting alignment was exported in the "msf" format and imported and analysed in the MEGA 2 software package (Sudhir Kumar, Koichiro Tamura, Ingrid B. Jakobsen, and Masatoshi Nei (2001) MEGA2: Molecular Evolutionary Genetics Analysis software, Bioinformatics (submitted). This software is available as freeware from http://www.megasoftware.net/. The resulting dendrogram is shown above

Selection of primers and development of a PCR detection method.

Based on the 16S rRNA gene sequence data in comparison to available sequence data a series of potential primers was designed. The primers designed are listed in Table 4:

Table 4: possible primer sequences and expected amplicon size (BBF-1 in combination with BBR-2, -3 or -4).

Annotation	Sequence	Direction	approx. Amplicon
BBF-1	5' TGAGTAATGCCTAGGAACA	forward	n.a.
BBR-2	5' CTAATCCCACCTAGGCCA	reversed	140 bp
BBR-3	5' TAACGTCAAGAGGCATGC	reversed	400 bp
BBR-4	5' TCTCCGCTGGCTTCTGTA	reversed	903 bp

Two sets of primers were made and tested in a basic PCR test. The chosen primer sets were BBF-1 with BBR-3 and BBF-1 with BBR-4 because of their expected amplicon size. In the initial test two BBS-strains; INS 445 and INS 600 (representing both countries of isolation) were used together with a *V. anguillarum* internal reference strain INS 198. Colonies were grown for 48 hours at 26°C on Choc+ agar for the BBS-strains and on TSA-1 for *V. anguillarum*. A small portion of an isolated colony was picked from the plate by means of a injection needle and transferred to a tube containing puReTaq Ready-To-Go PCR beads (Amersham Biosciences Cat no. 27-9558-01), approximately 1 µl of bacteria, 0.5 µl of each of the primers (10 µM stock solution) and 23 µl of double distilled water. The samples were then run using Hybaid Thermal cycler with the following settings:

Denaturation	95°C	2mins	x 1 cycle
Amplification	95°C	30secs	
	54°C	45 secs	x 30 cycles
	72°C	45 secs	
Extension	72°C	5mins	x 1 cycle

PCR products were analyzed on 1.2% agarose gel (BST Techlab Cat no.BST3001-100g) in TAE buffer (4.84g Trizma Base MW: 121.14, 3.09ml 0.5M EDTA, 1.142ml

Glacial acetic acid and to 1 liter of de-ionised water). The gels were generally run at 120V for 60mins, stained with ethidium bromide during gel run and photographed using SynGene Bio Imaging System.

In a subsequent PCR run the settings were further optimized by increasing the annealing temperature with 2°C, while leaving all other setting unaltered.

Results

The results for the first run are given in Figure 6.

The results from this first run indicated that both primer-sets yielded an amplicon of the expected size when applied on BBS-strains. However, both primers cross-reacted with the *V. anguillarum* strain and gave an amplicon of a different than expected size and of a marked lower yield.

In order to optimize the PCR we increased the annealing temperature with 2°C. This annealing temperature would theoretically favor the BBF-1/BBR-4 primer-set. All other methods and procedures remained unchanged.

The results of the second PCR run are presented in Figure 7. For this run, only one BBS-strain (INS 445) and the *V. anguillarum* internal reference strain (INS 198) were amplified.

The results show that the unwanted cross-reactions observed at a lower annealing temperature were gone, i.e. *V. anguillarum* did not yield a amplicon, however, the first primer set (BBF-1/BBR-3) gave a marked lower, yet specific yield as compared to the first run. Based on the results obtained the primer set BBF-1/BBR-4 was chosen as a specific primer set, with the additional advantage that the end PCR-product is easily detected in standard gel measurements, due to its size.

Evaluation of primer specificity

This experiment aimed at evaluating the specificity of the primers for BB. In order to achieve this a series of strains, representing different fish pathogenic and non-pathogenic species were tested. An overview of the species tested is given in table 5.

The choice of test-strains was determined by the 16S rRNA cluster analysis and phylogenetic tree establishment as well as by the most likely contaminants present in field samples. A total of 17 species were included in the comparative analysis.

The methods applied were identical to those described above. Only one primer set was used: BBF-1/BBR4, and the annealing temperature was kept at 56°C.

Table 5: List of species used in the specificity analysis of the PCR-detection method and the lane layout as presented in the gel.

Lane	Strain number	Species	Origin / host
1	SB 1129	Big Belly strain	*L. calcarifer*
2	NCIMB 1280	*Vibrio harveyi*	Reference strain
3	ATCC 43305	*Vibrio anguillarum*	Reference strain
4	NCIMB 1318	*Vibrio alginolyticus*	Reference strain
5	LMG 8893^T	*Lactococcus garvieae*	Type strain
6	LMG 14520^T	*Streptococcus iniae*	Type strain
7	CCUG 10073^T	*Nocardia asteroides*	Type strain
8	YT 961	*Photobacterium damsela* subsp. *damsela*	*S. quinqueradiata*
9	INS 602	*Photobacterium damsela* subsp. *piscicida*	*S. dumerilii*
10	DNA ladder		
11	RT 1202	*Flavobacterium columnarae*	*O. mykiss*
12	SB 1132	*Tenacibaculum maritimum*	*L. calcarifer*
13	ATCC 700383^T	*Pseudomonas plecoglossicida*	*P. altivelis*
14	INS 162	*Aeromonas salmonicida*	*S. salar*
15	ATCC 33907^T	*Aeromonas media*	Type strain
16	NCIMB 1895^T	*Photobacterium angustum*	Type strain
17	INS 242	*Edwardsiella tarda*	*A. japonicus*
18	INS 163	*Yersinia ruckerii*	*S. salar*

Results

The results of the specificity analysis are presented in figure 8.

As is apparent from figure 8, the PCR primer combination (BBF-1/BBR4, the annealing temperature kept at 56°C) as used is highly specific. Only the BBS-strain used gave a PCR product. All other species and genera did not.

Evaluation of available BBS-strains and detection limits

Representative isolates for the different strains of the bacterium according to the invention in the collection were tested. An overview of the strains tested is given in Table 6. The PCR test was performed as described above, with an annealing temperature of 56 degrees.

Table 6: Strains used for PCR testing

Strain code	Isolation origin	Country of isolation
INS 445	seabass	Indonesia
INS 454	seabass	Indonesia
INS 512	seabass	Indonesia
INS 527	seabass	Indonesia
SB 1128	seabass	Indonesia
INS 598	seabass	Singapore

In addition we evaluated the detection limits starting from pure growth. For this strain INS 445 was grown for 48 hours on Choc+ agar at 26°C. Growth was harvested in PBS (0.01 M, 15% NaCl (w/v), pH 7.2) and the number of cells was counted by using a Petroff-Hauser counting chamber. Subsequently, the bacterial suspension was adjusted to 1 x 10^8 cells per ml and serially ten-fold diluted. From the dilutions that contained 10^3, 10^2, 10^1, 10^0 and 10^{-1} cells per ml, 1 ml was centrifuged at 14,000 RPM for 10 minutes and the pellet was processed as described above.

Results

The results are presented in figure 9. The results clearly and unambiguously show that all BBS-strains are recognized to the same extend by the primers, further confirming their identity. In addition, the PCR test is able to detect as little as 10 cells as a minimal amount in the presently used program (number of cycles).

Legend to the figures:

Figure 1: Mortality over time of juvenile seabass in culture.

Figure 2: Giemsa-stained impression print of the spleen of Asian seabass showing bipolar appearance (double arrow) and the "vacuole" (single arrow).

Figure 3: EM picture of the intracellular organisms.

Figure 4: titers expressed as the reciprocal of the serum dilution required to obtain 0.4 reading in the ELISA

Figure 5: Mortality caused by BBS-bacterium in challenged and control fish.

Figure 6: PCR results for two primer sets against two BBS-strains and a *V. anguillarum* internal reference strain. Lanes: 1; INS 445; 2; INS 600; 3; INS 198; 4; INS 445; 5; INS 600; 6; INS 198; 7; DNA ladder. Primer set used: BBF-1/BBR-3 (lanes 1-3) and BBF-1/BBR-4 (lanes 4-6). $T_{amplification} = 95/54/72^0C$.

Figure 7: PCR results for two primer sets against a BBS-strain and a *V. anguillarum* internal reference strain. Lanes: 1, INS 445; 2, INS 198; 3, DNA ladder; 4, INS 445; 5, INS 198. Primer set used: BBF-1/BBR-3 (lanes 1-3) and BBF-1/BBR-4 (lanes 4-6). $T_{amplification} = 95/56/72^0C$.

Figure 8: Specificity of the BBS-PCR against different aquatic bacteria. The relation between lanes and tested strains is represented in table 5. Primer set used: BBF-1/BBR-4

Figure 9: PCR results for the different strains of BBS, isolated at different time points and different locations; and detection limits for the PCR-reaction. Lanes: 1: INS 445; 2: INS 598; 3: INS 512; 4: INS 527; 5: INS 454; 6: SB 1128; 7: DNA ladder; 8: INS

445 10^3 bacteria; 9: INS 445 10^2 bacteria; 10: INS 445 10^1 bacteria; 11: INS 445 10^0 bacterium; 12: INS 445 10^{-1} bacteria; 13: INS 198.

Claims

1) Gram-negative pleiomorphic rod-shaped bacterium causing Big Belly disease in fish, characterized in that said bacterium comprises a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1 or characterized in that the bacterium comprises a 16 S rRNA gene that reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/- 15 base pairs.
2) Gram-negative pleiomorphic rod-shaped bacterium causing Big Belly disease in fish, characterized in that said bacterium comprises a 16 S rRNA gene of which the nucleotide sequence has a level of identity of at least 97 %, preferably 98 %, more preferably 99 % and most preferably 100 % to the nucleotide sequence as depicted in SEQ ID NO 1 and characterized in that the bacterium comprises a16 S rRNA gene that reacts in a PCR reaction with primers as depicted in SEQ ID NO 2 and 3, to give a PCR product of 903 +/- 15 base pairs.
3) Gram-negative pleiomorphic rod-shaped bacterium according to claim 1 or 2, characterized in that said bacterium is in a live attenuated form.
4) Gram-negative pleiomorphic rod-shaped bacterium according to claim 1 or 2, characterized in that said bacterium is in an inactivated form.
5) Microbiological culture comprising a bacterium, characterized in that said culture comprises a bacterium according to claims 1-4.
6) Microbiological culture according to claim 5, characterized in that said culture comprises NAD.
7) Vaccine for combating Big Belly Disease in fish, characterized in that said vaccine comprises a bacterium according to claims 1-4 and a pharmaceutically acceptable carrier.
8) Vaccine according to claim 7, characterized in that said vaccine comprises at least one other microorganism or virus that is pathogenic to fish, or one other antigen or genetic material encoding said other antigen, wherein said other

antigen or genetic material is derived from a virus or microorganism pathogenic to fish.

9) Vaccine according to claim 8, wherein virus or microorganism pathogenic to fish is selected from the group consisting of the fish pathogens *Vibrio anguillarum, Photobacterium damselae* subspecies *piscicidae, Tenacibaculum maritimum, Flavobacterium sp., Flexibacter sp., Streptococcus sp., Lactococcus garviae, Edwardsiella tarda, E. ictaluri,* Viral Necrosis virus, iridovirus and Koi Herpesvirus.

10) Vaccine for combating Big Belly Disease, characterized in that said vaccine comprises antibodies specific to a bacterium according to claims 1-4 and a pharmaceutically acceptable carrier.

11) Vaccine according to claims 7-10, characterized in that said vaccine comprises an adjuvant.

12) Antibody specific to a bacterium according to claims 1-4.

13) Bacterium according to claims 1-4, for use in a vaccine.

14) Use of a bacterium according to claims 1-4, for the manufacture of a vaccine for combating Big Belly Disease.

15) Method for the preparation of a vaccine according to claims 7-9, characterized in that said method comprises the mixing of a bacterium according to claims 1-4 and a pharmaceutically acceptable carrier.

16) Method for the preparation of a vaccine according to claim 10, characterized in that said method comprises the mixing of antibodies reactive with a bacterium according to claims 1-4 and a pharmaceutically acceptable carrier.

17) Diagnostic test kit for the detection of antibodies reactive with a bacterium according to claims 1-4, characterised in that said test comprises a bacterium according to claims 1-4, or antigenic material thereof.

18) Diagnostic test kit for the detection of a bacterium according to claims 1-4 or antigenic material thereof, characterised in that said test comprises antibodies reactive with a bacterium according to claims 1-4.

19) Diagnostic test kit for the detection of a bacterium according to claims 1-4, characterised in that said test comprises PCR-primers that specifically react with the 16 S rRNA gene of a bacterium according to claims 1-4.

20) Diagnostic test kit according to claim 19, characterised in that said test comprises PCR-primers as depicted in SEQ ID NO.: 2 or 3.