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Anthrax – a natural disease and a biological weapon

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Introduction

The bacterium that causes anthrax, *Bacillus anthracis*, has been with us throughout recorded history. The organism is unique because it is the only aerobic spore forming bacterium that appears to be an obligate parasite. It has been suggested that the organism has a natural cycle of replication within the soil[1] but there is no evidence that this occurs consistently, if at all, because the nutrient supply is limited and the environment is hostile. However when an animal dies of anthrax and the carcass is consumed by carnivores the release of large quantities of nutrient-rich body fluids containing huge concentrations of anthrax organisms might encourage the organism to replicate in the soil,[1] especially in warm conditions. Eventually, when the nutrient supply declines, the bacteria will produce the resistant spores that are the major reason for the persistence of the disease in the wild. Animals that have died of anthrax can cause considerable contamination of the local area, becoming a source of infection particularly for browsing herbivores[2]. Vultures and other scavengers, which are relatively resistant to anthrax infection can, in their droppings, disperse the agent over vast distances. Although anthrax is enzootic in many regions of the world, it is in Africa that the problem is particularly acute. The huge herds of herbivores graze every square metre of grassland and it is inevitable that some of them will consume an infectious dose of the anthrax spores lying dormant close to the surface of the soil. Although anthrax is not a contagious disease the natural cycle of infection, death, disruption of the carcass by scavengers and the dispersal of the spores can produce an enormous increase in the number of animals infected. The consequent decline in the local animal population can be to the point of annihilation in some instances. The role of the scavengers in this cycle of infection is critical because without their activity the carcass of the fallen animal will remain undisturbed, without the anthrax bacteria becoming exposed to oxygen and forming spores. The non-sporulating bacteria will then decay within a short time, thus ending the infectious cycle.

Infection in livestock

Fortunately in the United Kingdom deaths from anthrax in livestock are rare. Where the disease is suspected, veterinary surgeons are only allowed to take a small sample of blood from a convenient site, such as the ear, for examination under a microscope. After staining the smear with Macfadyean's stain the observation of large blue bacilli surrounded by a pink capsule (**Figure 1**) is characteristic of anthrax and the diagnosis is irrefutable. If the Macfadyean's test proves positive the carcass should be destroyed by incineration[3]. If this is not possible it should be buried at a depth of at least six feet sandwiched between layers of lime[4]. In developing countries such precautions are not observed, especially when the infection spreads to herds of domestic cattle. A dead cow represents a considerable economic loss to a rural herdsman and consequently the carcasses of dead animals, regardless of the cause of death, are dismembered and offered for sale.

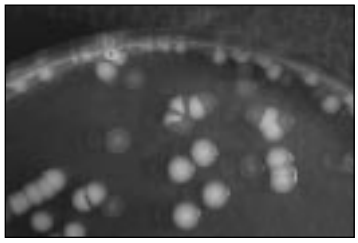
Infection in humans

Only thorough cooking of contaminated meat at temperatures in excess of 100°C will kill the spores[5,6] but it is inevitable that some meat will be consumed semi-cooked. In this case live spores and even live vegetative bacteria may be consumed in enormous quantities, far in excess of the minimum infectious dose by the gastro-intestinal route[7]. Once infection has been established in the alimentary tract death has always been inevitable.

The handling of animals that have died of anthrax creates the further risks of cutaneous infection where the spores penetrate the skin through cuts and abrasions. This is the most common form of infection and the least dangerous[8]. The spores germinate in the dermis and establish an infection that may remain localised throughout its existence. After an incubation period of two to three days a small red pimple appears. Over the next day or so this becomes surrounded by a ring of vesicles. The pimple ulcerates and dries, producing

Also in this issue (page 5):

Vibrio vulnificus



Anders Dalsgaard
Opaque and translucent colonies of *V. vulnificus*.

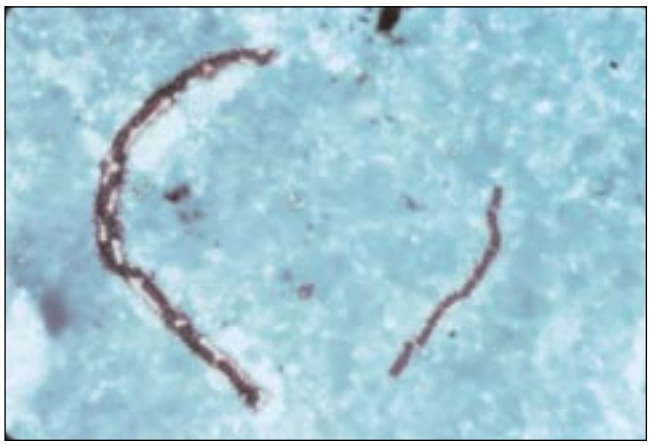


Figure 1. Macfadyean's stain showing the characteristic blue anthrax cylindrical bacilli surrounded by the pink polyglutamic acid capsule.

the characteristic black eschar (**Figure 2**). The whole of this complex lesion is called a malignant pustule but it exhibits neither of these properties. The anthrax organisms can readily be isolated from the lesion by carefully lifting the eschar away from the underlying tissues and taking a swab sample from beneath it. On culture the swab will produce the characteristic colonies of *B. anthracis* on blood agar (**Figure 3**). As it develops the lesion becomes surrounded by very extensive swelling or oedema. In spite of its dramatic manifestations the infection causes little or no pain to the patient unless secondary infections occur. In the developed world cutaneous infections do not usually result from handling a recently deceased animal but are derived from imported animal products from countries where anthrax is enzootic. At one time controls on such imports were few and there were classic cases resulting from the use of shaving brushes which had been manufactured from animal hair contaminated with anthrax spores; the brush and the razor being a potentially lethal combination. Contaminated bone meal is also a source of cutaneous infection[9] but by far the most serious infection is caused by the processing of contaminated hides and hair. Such procedures are associated with copious quantities of dust which is inhaled by the work force. If the hides and wool are contaminated, spores of *B. anthracis* will also be inhaled. Clinical anthrax caused by the inhalation of spores does not always occur and depends on a number of factors such as the general health of the host, the virulence of the organism and the number of spores inhaled.

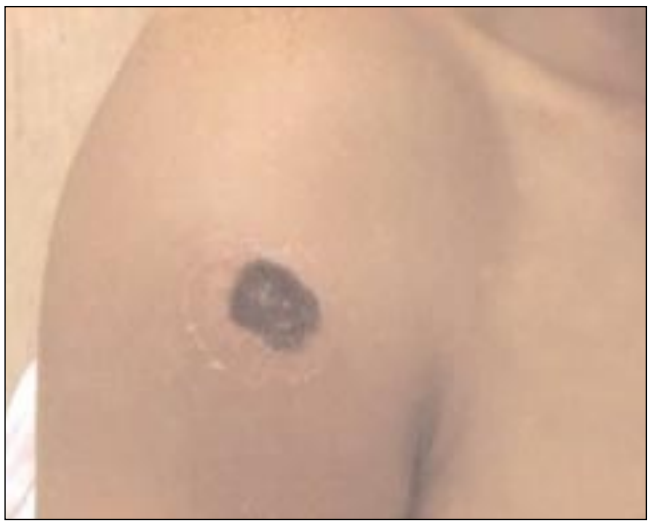


Figure 2. The typical malignant pustule of the cutaneous infection showing the central black eschar. (Centers for Disease Control, Atlanta, GA, USA).

Some individuals may succumb from exposure to only a few hundred spores while others may tolerate a dose of many thousands. In a pioneering experiment in the USA the air from a woollen mill was diverted through an enclosure in which monkeys were housed. A number of these animals subsequently died from inhaled anthrax. By contrast the work-force which had been breathing the same air seemed totally unaffected, a result which confirmed the different susceptibilities of different species[10]. This industrial disease, usually called "Woolsorters Disease"[11] was not uncommon in the United Kingdom during the early part of the last century but due to the imposition of import controls, use of dust masks, and compulsory sterilisation of imported animal products it is now virtually unknown. The course of all systemic infections is of insidious onset, sometimes with mild fever and malaise, but this initial stage is followed by a sudden and catastrophic acute condition with dyspnoea, cyanosis, high fever, coma and death after a few hours. In the past certain death was the immutable outcome for a patient presenting with symptoms of systemic anthrax. However recent events in the USA have shown that even if victims present with early symptoms the death rate can be markedly reduced[12].

The development of anthrax as a biological weapon

The use of spores of *B. anthracis* as a biological weapon had relatively small beginnings that have only recently come to light[13]. It had been known since the 1880s that anthrax was caused by a bacterium that grew readily in culture and produced resistant spores that would survive for decades. Towards the end of the World War I the German intelligence services tried to exploit the properties of the organism as a weapon of sabotage. They used the services of Baron Otto Karl Robert von Rosen, a Swedish-German-Finnish aristocrat. In the winter of 1917 the Baron and his companions were arrested in the remote Finnmark area of Norway. When the Baron's luggage was searched, among the usual items of explosives, there were bottles of curare, "microbial cultures" and 19 sugar cubes each enclosing a tiny glass tube containing anthrax spores. The Norwegian newspapers at the time were convinced that the contaminated sugar cubes were to be used to disrupt the transportation of merchandise by horses and reindeer between Skibotten and the Finnish border. The grinding of the sugar with its glass insert between the molar teeth of the animals is likely to result in a lethal infection as the anthrax spores gain access to the body, facilitated through the small lesions caused in the wall of the alimentary tract by the broken glass.

Although this episode had long been forgotten by the time that World War II was imminent, the British authorities believed that they should be capable of mounting a retaliatory response should biological weapons be used by Germany. Anthrax was the obvious candidate and large quantities of the spores were produced at Porton Down. These spores were incorporated into millions of small cakes of cattle feed that could be released over farming areas of Germany to kill livestock; a trial of the weapon proved that the cakes were indeed capable of causing a lethal infection in cattle and horses. However this was not the anti-personnel weapon that the military required and top secret experiments were instigated to produce an anthrax bomb which would produce an airborne cloud of spores that would be inhaled in sufficient

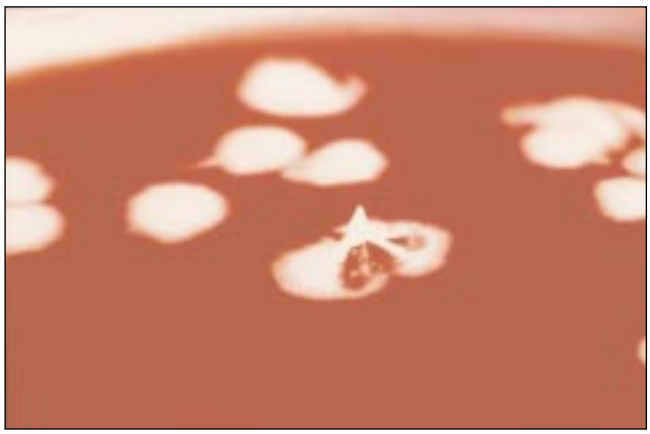


Figure 3. Colonies of *B. anthracis* on blood agar. Note how the colony forms a stable extension when touched with an inoculation loop. (Hodder-Arnold Ltd)

numbers to produce a lethal infection in man. It had been shown in laboratory experiments at Porton that animals could be infected by the aerosol route but it was now necessary to carry out trials in the field but in a location that was remote enough to reduce the risks to humans and animals to the absolute minimum.

Eventually, the Scottish island of Gruinard was chosen. It was close enough to the mainland to allow easy access and it was free from the threat of enemy action. It was also in an area with a sparse population, the nearest town being Ullapool some 12 miles to the east. The trials in 1942 and 1943 were performed on a relatively flat saddle of land between 2 high points towards the southern end of the island approximately 50m above sea level. Mostly they involved placing a 4lb bomb, containing a heavy suspension of spores of *B. anthracis*, on the ground and detonating it electrically. The bombs were cylindrical with a central rod-shaped explosive charge surrounded by the slurry of anthrax spores; the whole was encased by a thin metal skin. When the bomb was detonated the anthrax spores were dispersed radially. However only a small percentage of the fluid weapon-fill was dispersed as an airborne cloud with particles in the respirable size range of 1–5µm. The rest was scattered as large globules of spore slurry over the ground in the immediate vicinity of the detonation point and for a short distance downwind. The results were conclusive because all those sheep within the footprint of the airborne cloud died of anthrax except those on the periphery of the cloud where the inhaled dose was insufficient to induce infection[14]. Although the lethal consequences of exploding an anthrax bomb had been shown, full development of the weapon in the UK was never completed and the war ended with the project very much in a state of uncertainty. Towards the end of the decade the concept of dispersal of biological weapon agents, including anthrax, from a spray device rather than by explosive means began to hold sway and a number of trials at sea, both off the coast of Scotland and in the Caribbean, were successfully carried out.

In the USA however, development of explosive dispersal weapons continued and eventually vast numbers of bombs were produced and stockpiled[15]. These contained not only anthrax spores but also other candidate biological weapon agents. In the UK throughout the 1950s and early 1960s biological weapons policy continued to drift and eventually it became wholly defensive in nature. In 1969 President Richard Nixon terminated biological weapons research in the USA entirely and in the mid 1970s in the UK a panel of independent experts decided that the threat from biological weapons was "nebulous". As a consequence, in 1979, ownership of the Microbiological Research Establishment (MRE) was transferred to the Public Health Laboratory Service under a new name.

It was ironic that almost to the day that MRE closed, an accidental release of an aerosol of anthrax spores from a biological weapons facility at Sverdlovsk in the former Soviet Union caused many human deaths[16]. When this outbreak of human anthrax was revealed, the Russian Authorities said that the cause was the consumption of undercooked contaminated meat but the whole truth only emerged after the collapse of the Soviet Union. It seems extraordinary that western intelligence had no convincing information concerning the huge biological weapons research and development programme that was taking place behind the Iron Curtain over several decades. The extent of the Russian programme was not realised until high profile defectors and inspections by western experts revealed the full range of its activities[17].

However, in August 1990 a new threat had emerged in the Middle East. Iraq had already used chemical weapons against Iran and against dissident elements in its own population. There was also intelligence that Iraq had a significant programme to develop weapons of mass destruction including biological weapons. Coalition forces carried out an extensive bombing campaign to reduce the chemical and biological weapons facilities to rubble but when United Nations inspection teams entered Iraq they found that at least one major facility with large scale fermenters capable of producing biological weapons had not been targeted for bombing[18]. In fact the UN inspectors never found large stockpiles of biological weapons but eventually the Iraqi government confirmed that they had produced but had then destroyed enough anthrax spores and botulinum toxin to mount a limited biological attack. A number of other countries are also though to be developing a biological weapons capability. These new threats have given a considerable impetus to biological weapons defence programmes and the particular threat of anthrax remains high on the list of candidate agents. The recent events involving the release of small quantities of powdered anthrax in the American Media and Legislature[19] has brought the wheel full circle from the foiled sabotage of Baron von Rosen in 1917 to the recent more successful sabotage in the USA. To counter this emerging threat, huge funds, for research on anthrax, have been made available to government laboratories and academic establishments, particularly in the USA[20]. It is likely that more than 90% of the current research on anthrax relies on military sources for its funding. The fruits of this extra funding are likely to be more reliable and rapid methods of detection and identification and more effective therapy and prophylaxis; all essential from a military standpoint.

Therapy and prophylaxis

Fortunately *B. anthracis* is sensitive to a number of antibiotics, particularly penicillin, doxycycline and ciprofloxacin although penicillin-resistant isolates have been reported. Using genetic engineering techniques Russian workers have also constructed a vaccine strain of anthrax which is resistant to multiple antibiotics[21]. At present natural isolates showing multiple resistance have not been discovered. Antibiotic therapy is very effective in the early stages of infection, particularly against the

cutaneous form of the disease. It has now been shown that even patients presenting with early stage symptoms of inhalational anthrax can be saved by intensive antibiotic therapy and aggressive supportive care[12]. However, if the symptoms are not recognised early, antibiotic therapy is unlikely to prevent a fatal outcome. Even if the blood is sterilised by large doses of antibiotic the lysing bacteria release such large quantities of toxin that the victim succumbs. As a precaution it is worthwhile to offer a course of vaccination to patients to prevent recurrence of infection once antibiotic therapy has been terminated.

Anthrax vaccines have been available for more than 100 years and although Louis Pasteur is given the credit for the first attenuated live vaccine, in reality the honour should go to W S Greenfield who had previously developed a similar vaccine[22]. However, it was the Pasteur vaccine that was adopted for widespread use over the next 50 years. Although it was effective it had a short shelf life and it varied so much in virulence that particularly susceptible species were at risk of dying from the vaccine itself. As the disease in animals was so important the occasional infections in man were considered to be tolerable and there was no vigorous effort to develop a vaccine for human use. The shortcomings posed by the Pasteur vaccine were not resolved until the 1930s when an effective attenuated spore vaccine was developed for use in animals[23]. This vaccine is still in use today but animals must be re-vaccinated every year to maintain effective levels of protection. Similar attenuated live spore vaccines for human use have been limited to Russia and China[24,25]. Such vaccines although providing good protection also produce severe side effects and are considered unsuitable for use in western nations.

The awareness of the threat from anthrax as a biological weapon after the Second World War stimulated the United Kingdom to develop an effective vaccine which was not based on attenuated living bacteria but on a component of the toxin complex elaborated by the organism. It had been known since 1904 that fluid taken from the oedema produced by an anthrax infection had immunising properties but it was not until the 1950s that the triple component nature of the extracellular anthrax toxin was elucidated[26]. One of the components of the toxin was termed the protective antigen (PA) and it was on this that the UK chemical vaccine was based. The vaccine preparation is relatively crude consisting of protein precipitation from anthrax culture which has been grown in such a way that the production of the undesirable components of the toxin is minimised. A similar vaccine is produced in the USA. There were some concerns about the protective efficacy of the vaccine which was shown not to be particularly effective in invoking protection in guinea pigs against an aerosol challenge. However more recent experiments in the USA have shown persistent protection in monkeys against an aerosol challenge after only two injections of the vaccine[27]. Unfortunately the vaccine produces some unpleasant side effects in some individuals such as inflammation and swelling at the site of the injection and a general feeling of malaise.

The relatively crude nature of the chemical vaccine coupled with its tendency to cause undesirable reactions were such that it was decided to develop a more effective product which induced minimal or no side effects. The research and development work has now been going on for nearly two decades and in spite of the application of the most modern techniques of immunology and genetic engineering a new vaccine is not imminent. Understandably many avenues of research have been explored in a number of countries to produce an effective and well-tolerated vaccine. The failure of scientific research to deliver a usable product in spite of the generous funding it has received has been a cause of considerable frustration in official circles. Indeed the recent re-emergence of the anthrax threat has obliged the military authorities in the USA to order large stocks of the old-style chemical vaccine that produced so many complaints about its side effects when it was used to immunise troops during the 1990/91 Gulf War. In 2001 the 4th International Anthrax Conference took place and there were nine separate proposals for a new anthrax vaccine. It is surely time to review the progress that has been made and to make a decision about the most promising technology to pursue. There is now an opportunity for all the countries involved to come together and agree a way forward for the next generation anthrax vaccine based on sound scientific principles rather than on professional rivalry and nationalistic pride.

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Vibrio vulnificus

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Introduction

Vibrio vulnificus is a halophilic marine vibrio and opportunistic human pathogen that can cause severe wound infections and septicaemia with mortalities for cases of septicaemia as high as 50 percent. The first case of *V. vulnificus* infection was most likely reported in the 5th century BC by Hippocrates. The king of the island Thasos in the Aegean Sea contracted an acute infection which was characterized by a swollen foot with red and black skin lesions, rapidly progressive septicaemia, and death on the second day. It is proposed that this infection was caused by *V. vulnificus*.

Most *V. vulnificus* infections have been reported from the United States, Japan and Taiwan although a number of cases in Holland, Israel, Belgium, Germany, Sweden and Denmark have been published. In the United States, septicaemia with *V. vulnificus* is nearly always associated with the consumption of raw oysters and is responsible for about 90% of all seafood-related deaths[1,2]. Following contact with seawater or fish or shellfish, *V. vulnificus* can also cause wound infections which often require surgical debridement of the infected tissue and/or amputation. The bacterium has less often been isolated from patients with gastroenteritis and its role as a primary cause of gastrointestinal disease remains to be determined.

This article will mainly contain information about the food-borne illness (primary septicaemia) caused by *V. vulnificus*.

Ecology

V. vulnificus can be isolated from a wide variety of aquatic, mainly estuarine, ecosystems. The presence of the organism is favoured by high temperatures (>20°C) and intermediate salinities (15-25%). *V. vulnificus* has been reported in several areas of South Korea and the United States, with a study of the entire US East Coast showing that approximately 1% of the culturable vibrios were identified as *V. vulnificus*[3]. In temperate areas, *V. vulnificus* is less abundant than in subtropical waters, but has been isolated from coastal waters or implicated in human infections during the summer months in Denmark, Sweden, Germany, Holland and Belgium. Isolation of *V. vulnificus* from the Mediterranean has rarely been reported, probably because the high salinity (35%) inhibits the growth of *V. vulnificus*. Thus, clinical cases do not appear to have been reported from this area despite the millions of tourists who swim in the Mediterranean each year and the large volumes of oysters that are consumed. It is likely that *V. vulnificus* is present in very low concentrations in these waters because of the high salinity, and that the concentrations are too low to cause human infections.

Oysters, clams, mussels, fish, plankton, as well as water and sediment have all been described as reservoirs and vehicles for *V. vulnificus*. During the summer period on the United States Gulf Coast, DePaola *et al.*[4] isolated much higher (2 to 5 logs) numbers of *V. vulnificus* in estuarine fish than in the surrounding water, sediment, or nearby oysters and crustaceans. Highest densities were found in the intestinal contents of certain bottom-feeding fish (10^8/100g), particularly those that consumed molluscs and crustaceans[4]. *V. vulnificus* has also caused disease in eel farms in Japan, Spain, Norway, Sweden and Denmark. *V. vulnificus* has been isolated from waters with temperatures from 7°C to 31°C and salinities between 1 to 35% and is abundant in water with temperatures above 20°C and salinities between 15 to 25%. Sunlight, pH, nutrient factors and the presence of competing bacterial populations, including grazing, will also affect the distribution of *V. vulnificus* in the environment. There is no correlation between the prevalence of *V. vulnificus* and faecal bacterial indicators.

V. vulnificus in oysters

Since consumption of oysters containing *V. vulnificus* can cause primary septicaemia, several investigations, mainly in the United States, have been studying the occurrence and survival of *V. vulnificus* in oysters. During commercial harvest, oysters are typically held on the deck of the harvest vessel without refrigeration or icing until the vessel docks. Cook[5] found that *V. vulnificus* failed to multiply in oysters kept at 13°C or below for 30 h, whereas bacterial numbers were significantly higher when oysters were held at 18°C or higher. This and other studies indicate that normal occurring *V. vulnificus* can multiply in un-chilled oysters. It is therefore clear that a reduction of the time oysters are kept without refrigeration can decrease consumer exposure to high numbers of *V. vulnificus*. But oysters must be cooled immediately after harvest to eliminate post-harvest growth of *V. vulnificus*.

Taxonomy, isolation and identification

Taxonomy

V. vulnificus is a Gram-negative rod, aerobic and facultatively anaerobic, motile by means of a polar-sheathed flagellum, and is oxidase and catalase positive. *V. vulnificus* has been described as a "lactose-positive vibrio" or "L+vibrio", since the ability to ferment lactose was one characteristic that could distinguish this species from *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. However, it is now known that lactose fermentation is negative in up to 25% of the *V. vulnificus* isolates. The name *V. vulnificus* was given official taxonomic status in 1980. It is most likely that *V. vulnificus* in the past was often misidentified as *V. parahaemolyticus*. The species *V. vulnificus* consists of two bio-groups, which in the original definition differed phenotypically, serologically and in host range[6]. *V. vulnificus* biogroup 1 is ubiquitous in estuarine environments and is an opportunistic human pathogen. Biogroup 2 is typically recovered from diseased eels, but can also cause wound infections in humans after handling eels. The division into biogroups has been questioned and a division into serovars has been suggested[7]. This article discusses mainly the originally described biogroup 1, which is the major food-borne human pathogen.

Pre-enrichment and selective media

Pre-enrichment procedures often give improved recovery of *V. vulnificus* compared to plating on selective media although the choice of procedure should always be dependent on the sample type. The isolation of pathogenic *Vibrio* spp. is normally done by methods that include an initial pre-enrichment in alkaline peptone water (APW; 1% peptone, pH 8.6 with 1% NaCl) to recover sub-lethally injured organisms, followed by plating onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Early studies of *V. vulnificus* in environmental and clinical samples used this protocol although it was not optimized for the isolation of *V. vulnificus*. Various enrichment broths and selective agars have subsequently been tested for their capability of isolating *V. vulnificus*, The use of APW in combination with cellobiose-polymyxin B-colistin (CPC) agar and modified CPC (mCPC) agar has been reported to be effective in recovering *V. vulnificus* from oyster and water samples[8-13]. Sun and Oliver[13] found 82% of colonies (with correct morphology) of over 1000 colonies probed with a haemolysin gene probe to be *V. vulnificus*.

Arguments for using both polymyxin B and colistin in a *V. vulnificus*-selective agar have not been provided. Colistin and polymyxin B are both fatty acyl decapeptide antibiotics with bactericidal activity against most Gram-negative bacteria and are known by the name "polymyxins". Høi *et al.*[14] studied a collection of *V. vulnificus* strains for their sensitivity to colistin and proposed a new medium termed cellobiose colistin (CC) agar. CC agar gave a better *V. vulnificus* recovery than TCBS, CPC and mCPC agar in laboratory studies with pure cultures and with Danish water and sediment samples. The recovery rate on CC agar was significantly better than on mCPC agar[14]. The confirmation rate of presumptive isolates from CC agar was as high as previously reported for mCPC (approximately 95%) when taking into consideration the typical colony morphology of *V. vulnificus* on this medium (flat, yellow colonies of approximately 2 mm in diameter)[14,15]. In general, TCBS agar has been found to give very low plating efficiencies of both clinical and environmental *V. vulnificus* strains and can therefore not be recommended for the isolation of *V. vulnificus*. At present, CPC, mCPC, or CC agars are not commercial available.

Identification by serology and DNA-based methods

An enzyme immunoassay in an ELISA format has been recommended to identify presumptive *V. vulnificus* subcultured from mCPC agar[16]. The assay uses a *V. vulnificus*-specific monoclonal antibody (MAb) directed against an intracellular epitope of *V. vulnificus*.

DNA-based methods, particularly specific oligo-nucleotide probes used in colony hybridisation, represent a very sensitive and specific tool for identifying single *V. vulnificus* colonies or multiple colonies, e.g. by colony lifts from agar plates onto hybridisation membranes. An alkaline phosphatase-labelled oligonucleotide probe directed towards the cytolysin gene of *V. vulnificus* was made by Wright *et al.*[17] (probe sequence: GAGCTGTCACGGCAGTTGGAACCA). This probe demonstrated 100% specificity and sensitivity for clinical and environmental isolates of *V. vulnificus*. Numerous investigators have shown that cytolysin is produced by all *V. vulnificus* strains, including both biogroups, and is species-specific. The sequence of the cytolysin gene has also been used for constructing primers for PCR identification. More recently, it has been proposed that PCR primers or DNA probes directed against rRNA genes should be used in the identification of *V. vulnificus* since rRNA molecules are essential constituents of all living organisms and are present in growing cells in very high numbers[18]. The argument posed in favour of targeting rRNA genes is that a non-essential gene, such as the cytolysin gene, could theoretically be lost or re-arranged without affecting the viability of the bacteria.

Pathogenicity

Infection with *V. vulnificus* belonging to biogroup 1

V. vulnificus causes both food-borne and wound infections throughout the world. In the United States, it carries the highest death rate of any food-borne disease agent with approximately 50 cases per year requiring hospitalization.

V. vulnificus is highly invasive, causing fulminating primary septicaemia in persons at risk of infection, with mortality rates of approximately 50%[1]. Infection resulting in primary septicaemia is associated with consumption of raw shellfish containing the bacteria, especially raw oysters, with symptoms typically developing within 24 hours of ingestion. *V. vulnificus* infection following the consumption of other types of seafood is extremely rare. Death may occur within hours of hospital admission. Immunocompromised individuals or persons with elevated serum iron levels, typically a result of liver diseases such as cirrhosis or viral hepatitis, are at the highest risk for infection. Infections most frequently occur in males and it was shown that 82% of the cases reviewed were males with an average age exceeding 50 years[1]. There are several possible explanations why males are at higher risk for *V. vulnificus* infections than females. Firstly, males seem in general more likely to take and accept health risks (e.g. eating raw oysters) than females. Further, males consume raw oysters more often and in higher numbers than females. Although yet unknown, there may be sex-dependent genetic host factors that could explain the differences in susceptibility to infection. This is supported by findings that male rats showed a significant higher mortality than female rats when injected intravenously with *V. vulnificus* LPS. Common symptoms shown by patients with the primary septicaemia form of infection include fever (94%), chills (86%), nausea (60%) and hypotension (systolic pressure <85mm; 43%). An unusual symptom shown by more that 50% of the patients is the development of secondary lesions, typically of the extremities, which often require surgical debridement and/or result in amputation[1].

In addition to the primary septicaemia that follows ingestion, *V. vulnificus* is known to infect wounds of otherwise healthy individuals. However, the majority of patients with serious wound infections have an underlying disease[1,19]. *V. vulnificus* infections occur most often as a result of contamination of pre-existing wounds with seawater or after contact with fish or shellfish. Wound infection symptoms include localized pain, oedema, and erythema, with possible severe necrosis of the surrounding tissue requiring surgical debridement or amputation[1]. Mortality rates following wound infection are approximately 25% [1,19]. In a review of 11 patients infected with *V. vulnificus* during an unusually warm summer in 1994 in Denmark, Dalsgaard *et al.* [20] reported that 4 developed bacteraemia, one of whom died, and 9 others

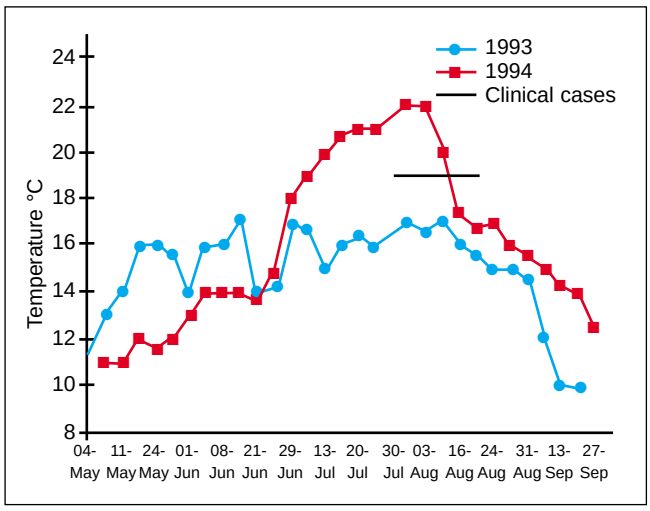

Figure 1. Mean water temperatures at three popular recreational beaches in Denmark. The bar shows the time period when the human *V. vulnificus* infections occurred in 1994 and 1995.

developed skin lesions. These infections and additional wound infections in 1995 were reported when water temperatures were above 20°C. **Figure 1** shows the mean water temperatures at four popular beaches in Denmark in 1993 and 1994 and the period during which the human cases of *V. vulnificus* were reported.[20]

Biogroup 2 strains

Whilst *V. vulnificus* is a pathogen for humans, Tison *et al.* [6] reported that certain strains isolated from locations in Japan were pathogenic for eels. Biogroup 2 strains have caused major disease problems in Danish eel culture[7,21]. This subset of *V. vulnificus* strains was termed biogroup 2, based on phenotypic differences from the human pathogens which comprise biogroup 1. Biogroup 2 strains have been shown to possess similar virulence factors as biogroup 1, including production of exo-proteins, uptake of various iron sources via phenolate and hydroxamate siderophores, and both LPS and capsule expression[22]. However, the lipopolysaccharides of biogroup 2 strains are homologous, which is different from those of biogroup 1 which are heterogenous. There is some evidence that *V. vulnificus* biogroup 2 strains can also cause human disease, e.g. wound infections. There have not been any human cases of *V. vulnificus* associated with the consumption of infected eels and such risks appear very low.

Virulence factors

A range of factors have been implicated as possible virulence determinants for *V. vulnificus*, including an extra-cellular haemolysin/cytolysin, an elastolytic protease, the ability to acquire iron from transferrin, the presence of a polysaccharide capsule and an endotoxic lipopolysaccharide, and resistance to the bactericidal effects of sera. A review of these putative virulence factors has been provided by Linkous and Oliver[23]. In addition, *V. vulnificus* strains can shift between virulent and avirulent forms, with virulent forms being encapsulated, serum resistant and possessing the ability to acquire iron from iron-saturated transferrin, while avirulent variants lack these characteristics[24].

Exoenzymes

V. vulnificus produces a large number of extra-cellular compounds, including haemolysin, elastase, collagenase, DNase, lipase, phospholipase, mucinase, chondroitin sulfatase, hyaluronidase, fibrinolysin and albuminase. More recent studies have shown that *V. vulnificus* protease is implicated in the production of bradykinin, which is an inflammatory mediator that increases vascular permeability, causes vasodilation and induces both pain and contraction of smooth muscle. Thus, proteases could be important in the intravascular dissemination of *V. vulnificus* and the development of septicaemia.

The haemolysin produced by *V. vulnificus* has been isolated, purified and shown to be lethal to mice when administered intravenously at low concentrations (3 µg/kg). Whilst the haemolysin has been shown to be produced *in vivo*, a lack of correlation between haemolysin production and virulence has been demonstrated.

Utilization of iron

Elevated serum iron levels seem to be a critical factor in the pathogenesis of *V. vulnificus* infections, with successful infection apparently requiring an increase in transferrin saturation. This was shown by Wright *et al.*[25], who directly correlated virulence with host iron availability. *V. vulnificus* does not seem able to grow in normal human serum, while injection of iron into mice prior to the injection of bacterial cells significantly lowers the LD_{50}.

V. vulnificus simultaneously produces both phenolate and hydroxamate siderophores, with the phenolate siderophore enabling virulent isolates to acquire iron from highly saturated transferrin. It has been shown that a mutagenised virulent *V. vulnificus* strain, which lost phenolate siderophore production, exhibited reduced virulence thereby confirming that iron acquisition is required for *V. vulnificus* virulence.

Production of capsule

The presence of a capsular polysaccharide (CPS) is the best studied virulence factor of *V. vulnificus* and is essential to its ability to cause human infection. It has been demonstrated that an "antiphagocytic surface antigen" enables virulent *V. vulnificus* strains to resist phagocytosis by human polymorphonuclear leukocytes. This antigen was subsequently shown by electron microscopy and ruthenium red staining to be an acidic polysaccharide capsule.

Studies of virulent and avirulent strains have shown a correlation between virulence and colony opacity (**Figure 2**). All virulent strains show an encapsulated, or opaque, colony type, whereas non-encapsulated, or translucent, cells are avirulent[24]. This supports the suggestion that the presence of capsule confers resistance to phagocytic activity. Encapsulated cells mutate at a very high rate (typically 10^{-2} to 10^{-3}) to produce non-encapsulated cells, with the loss of capsule correlating with loss of virulence. Reversion of translucent cells to opaque cells has also been shown in some strains, but at a very low ($<10^{-6}$) rate.

Endotoxin

The symptoms of *V. vulnificus* septicaemia, as well as the inflammatory response seen in patients with wound infections, are typical of the endotoxic activity of LPS molecules, suggesting this molecule could be a major virulence factor. It was demonstrated that intravenous injections of *V. vulnificus* LPS (400 µg/kg body weight) in mice caused mean arterial

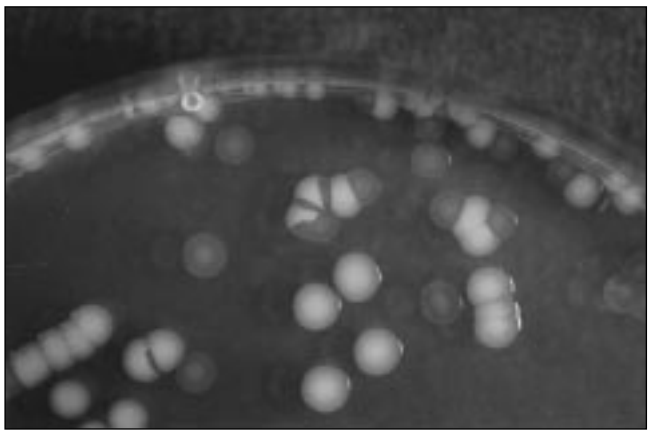

Figure 2. Opaque and translucent colonies of *V. vulnificus*.

pressure to decrease within 10 min, with death occurring in 30-60 min[26]. This was similar to the response seen when equivalent amounts of LPS from *Salmonella typhimurium* were injected.

Infectious dose and susceptible population

In the vast majority of *V. vulnificus* infections resulting from the ingestion of raw oysters, the patients have an underlying chronic disease. About 80% of these diseases are liver- or blood-related disorders, with liver cirrhosis secondary to alcoholism or alcohol abuse being the most typical. These diseases typically result in elevated serum iron levels, and laboratory studies have demonstrated that elevated serum iron plays a major role in infection with *V. vulnificus*. Other risk factors include haematopoietic disorders, chronic renal disease, gastric disease, use of immunosuppressive agents and diabetes.

The infectious dose of *V. vulnificus* is not known. However, Wright *et al.*[25] observed that in mice treated to produce serum iron overload, the LD_{50} decreased from 10^6 to a single cell. These and other data support epidemiological studies that indicate that liver damage, and often immuno-compromising diseases, are major underlying factors in the development of *V. vulnificus* infections. Further, the studies indicate that very low numbers of *V. vulnificus* may be needed to cause potentially fatal infections.

Antibiotic treatment and surgery

V. vulnificus is sensitive to the most commonly used antimicrobials. Various antimicrobials including tetracycline and third-generation cephalosporins have been recommended for the treatment of serious *V. vulnificus* infections. Dalsgaard *et al.*[20] demonstrated that several different antimicrobials were effective when wound isolates were studied *in vitro*. However, cases with serious wound infections often require surgical debridement.

Conclusions

V. vulnificus is implicated in both food-borne and wound infections throughout the world. Despite that, the bacterium is highly invasive, causing fulminate primary septicaemia with mortality rates of about 50% in susceptible individuals, infection is only associated with the consumption of raw shellfish, mainly oysters, containing the bacterium. Studies from the United States show that although oysters can contain high numbers of *V. vulnificus* (10^2–10^4 per gram) and millions of people are eating oysters, only about 30-50 human cases that have consumed shellfish are registered each year. Septicaemia does not seem to have been associated with the consumption of any frozen seafood products or fresh fish. Also, the role of *V. vulnificus* as a primary cause of gastrointestinal disease is questionable and remains to be determined. Analysis for *V. vulnificus* should therefore not be part of a standard seafood quality assurance program except for molluscan shellfish intended for raw consumption.

The occurrence of *V. vulnificus* is not indicated by the presence of bacterial indicators of faecal pollution as *V. vulnificus* is a normal inhabitant in mainly estuarine ecosystems with its occurrence being favoured by high temperatures (>20°C) and intermediate salinities (15-25%). Aquatic environmental and clinical strains show high degrees of heterogeneity in phenotypic and genotyping tests with little differences shown in their pathogenicity using experimental animals. However, the very low numbers of reported human cases compared with the number of persons exposed to high numbers of *V. vulnificus* through consumption of oysters suggest that all strains of *V. vulnificus* are not equally pathogenic or that not all individuals in the defined risk groups are equally susceptible.

Virulence is for certain dependent on the presence of an anti-phagocytic capsule and most likely also the endotoxin. Successful infection also seems to require certain host diseases, which predispose to *V. vulnificus* infections. Infection of these high risk individuals is associated with a very high fatality rate.

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