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Pathology and Etiology of Marine Mammals Causing Sudden Death in Republic of Korea

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ABSTRACT

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In Korea, there are wide range of marine mammals including spotted seals, Northern fur Seals, common dolphins and finless porpoises. Northern fur seals are usually found by one or two off the coast of Gyeongsangbuk-do, and spotted seals around Baengnyeong Island, the West Coast, the Southern Ocean in East and East Sea Coast area. There are also minke whales, Pacific white-sided dolphins, common dolphins, and Indo-Pacific bottlenose dolphins in the coast of Korea. Recently, the reports on marine mammal diseases increase worldwide. Although there are many marine mammals in Republic of Korea, a few study on the marine mammal diseases to date,

and thus we started to research on bacterial diseases of marine mammals. First of all, bacterial examination of the tissues of a male finless porpoise, *Neophocaena asiaeorientalis*, found dead in February 2010 in Tongyeong, Republic of Korea, was performed, and *Dietzia cinnamea* and *Clostridium tertium* were isolated from lung and intestine, respectively. *C. tertium* is a non-toxin producing clostridia, but damages gastrointestinal mucosa by direct colonization, and causes abscessation, osteomyelitis, and death in a dolphin. However, we could not determine whether these organisms resulted in any clinical symptoms of the finless porpoise. In the winter of 2012, an adult female Steller sea lion was found dead at Biyang-do, a small island near Jeju-do in Republic of Korea. As a result of bacterial examination, *Streptococcus phocae* and *Streptococcus halichoeri* were isolated, which were known as pathogenic bacteria of marine mammals. However, we could not prove if their infection contributed to the death of the Steller sea lion. A total of 44 long-beaked common dolphins, *Delphinus capensis*, 21 females and 23 males, were taken in the South and East Seas coast of Republic of Korea. They were bycaught in sotw nets on anchors from February 2012 to August 2013. Twenty-one spcies of bacteria were cultured from tissue samples of long-beaked common dolphins. Among the isolates, several bacteria are known as bacterial pathogen to marine mammals and humans. Histopathological examination could not reveal pathogenicity of the isolates on the death of dolphins. As a result of analysis of antimicrobial resistance in the bacteria isolated from marine mammals, *Streptococcus* spp. and *Staphylococcus* spp. were sensitive to all tested antibiotics. Fifty-four percent were resistant to at least one antibiotic, while 17% were resistant to multiple antibiotics.

Key words: antimicrobial resistance, bacteria, *Delphinus capensis, Eumetopias jubatus,* finless porpoise, long-beaked common dolphin, marine mammals, *Neophocaena asiaeorientalis,* Steller Sea Lion, Republic of Korea

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GENERAL INTRODUCTION

Diseases of emerging interest in marine mammals include infectious, neoplastic, environmental, anthropogenic and idiopathic. Bacterial disease is thought to be one of the leading natural causes of deaths in both wild and captive marine mammals. The prevalence and significance of most bacterial species isolated from many marine mammals are unknown.

Mass mortalities in marine mammals appear to be occurring with increased frequency worldwide. Various etiologies have been implicated, including bacteria, virus, and biotoxins. Each year, new bacterial species are discovered in cetaceans and pinnipeds, and some of them, such as *Brucella* spp., have the potential to infect people in direct or indirect contact with these animals. Some bacteria may be part of the normal flora in some marine mammals and are present in their environment. Some are opportunistic, causing disease when the animal is in some way compromised. For many, their clinical significance is unknown, since essential data, such as clinical history, clinical manifestations, and macroscopic and microscopic lesions, are not available.

In South Korea, there has been also increasing the report on mass deaths of marine mammals such as finless porpoises. Compared with Europe and the United States, relatively little research has been conducted on health and diseases in South Korea's marine mammals.

Historically, much of the pathologic data on marine mammal disease came

from marine mammal strandings, which is still a valuable source. More in-depth pathologic and epidemiologic data can originate from dedicated marine mammal population health assessment programs which use marine mammal species as sentinels for oceans and even human health. Such sentinels are used to gain early warnings about current or potential negative trends and impacts. Marine mammals are probably one of the best sentinel organisms in aquatic and coastal environments.

Microbial infection may contribute to disease in a significant proportion of marine mammal mortalities, but little is known about bacterial species in South Korea's marine mammals.

This study focuses on bacteria recovered from different tissues of marine mammals of Republic of Korea. These marine animals are including a stranded finless porpoise and a sea lion, and bycaught long-beaked common dolphins.

LITERATURE REVIEW

Introduction

Marine mammals are true mammals that have evolved unique characteristics for survival in an aquatic environment. There are five major groups of marine mammals: cetaceans (whales, dolphins and porpoises), pinnipeds (seals, sea lions and warlruses), sirenians (dugongs and manatees), sea otters and polar bears (Tryland et al., 2012). Based on sighting records, South Korea has diverse marine mammals including spotted seals, Indo-Pacific bottlenose dolphins, and common dolphins.

Marine mammals are sentinels for both ocean and human health because they have long life spans, feed at upper trophic levels, have extensive fat sotres, and are vulnerable to same pathogens, toxins, and chemicals as humans. They are another source of information about ocean environment. Their tissues can tell quantity of contaminant levels.

Cetaceans continue to mass-strand or mass-die, but the causes of the majority of these events remain unclear. Mass strandings or mass mortalities have received more attention as coastal human populations increase, making discovery of stranded animlas more likely.

Mass mortality events

The stranding of large numbers of marine mammals always commands a great deal of public and scientific curiosity. Although these events occur with greater frequency along certain coastlines, they can occur worldwide, posing questions about their causes and potential effects on human health. Many animals stranding at one time is referred to as a mass stranding. When many animals strand over an extended period of time, this is referred to as a mainre mammal unusual mortality event (UME). In the United States, there were stranding of endangered humpback whales (*Megaptera novaeangliae*) in 1987 (Geraci et al., 1989), and bottlenose dolphin (*Tursiops truncates*) die-off between 1987 and 1988 (Geraci et al., 1989). These evnets triggered the need for interested parties to develop a legal framework and subsequent law that addressed UME. In 1989, the Department of the Environment in the United Kingdom established a national program to investigate marine mammal mortalities in the United Kingdom and to coordinate responses.

In recent years, increased efforts to examine carcasses and live stranded animals have improved the knowledge of mortality rates and causes, allowing a better understanding of population threats and stressors and the ability to determine when a situation is unusual. Fifty percent or more sampled dolphins were positive for brevetoxin with most at high concentrations (Twiner et al., 2012). Similarly, past UMEs that have been attributed to morbillivirus

involved successful detection of morbillivirus in greater than 60% of dolphins tested (Krafft et al., 1995; Schulman et al., 1997). There is evidence that *Brucella*, which is commonly found in marine mammals worldwide, can cause disease in cetaceans, including bottlenose dolphins (Hernandez-Mora et al., 2013; Cassle et al., 2009; Dagleish et al., 2007; Davison et al., 2009; Miller et al., 1999). As such, there was a need to evaluate all of these potentially important diseases as playing contributing or leading roles in the ongoing UME. Understainding and investigating marine mammal UMEs is important because they can serve as indicators of ocean health, giving insight into larger environmental issues which may also have implications for human health and welfare.

The marine mammal UME program was established in 1991 in the United States. From then, there have been over 60 recognized UMEs, involving a variety of species and dozens to hundreds of individual marine mammals per event. In Republic of Korea, a mass mortality of 249 finless porpoise (*Neophocaena asiaeorientalis*) occurred at a dike of the Saemangeum Sea (Park et al., 2012) in 2011. From then, the events have been reported sporadically.

Causes of mortality events have included biotoxins, viruses, bacteria, parasites, human interactions, oil spills, and changes in oceanographic conditions (Table 1, Figure 1).

Stranded animals are a useful source of information on diseases occurring in

marine mammals, as they are more readily available to pathologists than free-ranging animals. A number of emerging disease entities, suites of lesions and infectious agents in marine mammals were first identified in stranded animals, after which their presence in the freeranging population was confirmed. Stranded animals can also alert us to diseases that are present in the more inaccessible wild animals that would be difficult to detect in random samplings of such populations (Gulland and Hall, 2007). Stranded animals, however, do not constitute an ideal system for the study of disease, as they do not represent the entire population (Aguilar and Borrell, 1994). To determine whether marine mammal health is indeed changing, a concerted effort is needed worldwide to coordinate investigations into marine mammal die-offs, to investigate associations between disease and ecological variables, to share data on methodology used for investigations and laboratory studies, and to document the stranding response effort.

Table 1. Marine mammal unusual mortality events since 1991

Year	Species	No. of animlas	Location	Cause
1991	Harbor seals	34	New York	Erysipelothrix rhusiopathiae, poxvirus
	California sea lions	160	California	Leptospirosis
	Bottlenose dolphins	30	Florida (Sarasota)	Unknown
1992	Harbor seals	100	Washington	Unknown
	Dugong	100	Queensland	Starvation
	Common dolphins	118	Southwest U.K.	Fisheries interaction
	Phocid seals	24	Massachusetts	Morbillivirus or influenza suspected
	Bottlenose dolphins	220	Texas	Carbamates suspected
1992-1993	California sea lions	1,000	California	El Nino
1993	Harbor porpoises	64	Massachusetts	Fisheries interaction
	Pinnipeds	53	Washington	Gun shot
1994	Common dolphins		Black sea	Morbillivirus
	Common dolphins	53	California	Unknown
	Bottlenose dolphins	72	Texas	Morbillivirus
1995	California sea lions	222	California	Leptospirosis
1996	Sea otters	68	Alaska	Malnutrition
	Right wahles	6	Florida	Blast injury suspected
	Manatees	149	Florida	Brevetoxin
	Bottlenose dolphins	30	Mississippi	Unknown

Table 1. Marine mammal unusual mortality events since 1991 (continued)

1997	Mediterranean monk seals	150	Western Sahara	Algal bloom, morbillivirus
	Harbor seals	90	California	Pseudomonas aeruginosa
1998	Hooker's sea lions		New Zealand	Unknown, bacteria likely
	California sea lions	70	California	Domoic acid
1999-2000	Bottlenose dolphins	115	Florida	Brevetoxin
2000	Caspian seals	10,000	Caspian sea	Canine distemper virus
	California sea lions	178	California	Leptospirosis
	Harbor seals	26	California	Viral pneunmonia suspected
2001	Bottlenose dolphin	35	Florida	Unknown
2001-2002	Hawaiian monk seals	11	Hawaii	Malnutrition
2002	Multispecies	500	California	Domoic acid
	Manatees	34	Florida	Brevetoxin
2003	Sea otters	69	California	Ecological factor
	Large whales	21	Gulf of Maine	Unknown
	Manatees	96	Florida	Brevetoxin
	Harbor seals and Minke whales		Maine	Unknown
2004	Bottlenose dolphins	107	Florida	Brevetoxin
	Small cetaceans	67	Virginia	Unknown
	Small cetaceans		Florida	Unknown
2005	Harbor porpoise		North Carolina	Unknown

Table 1. Marine mammal unusual mortality events since 1991 (continued)

	Large whales	North Atlantic	Unknown
2005-2006	Multispecies, Bottlenose dolphins	Florida	Brevetoxin
2006	Sea otters	Alaska	Unknown
	Humpback whales	North Atlantic	Unknown
	Pinnipeds	North Atlantic	Infectious disease
	Harbor porpoises	Pacific Northwest	Unknown
	Manatees	Florida	Biotoxin
2007	Bottlenose dolphins	Texas and Louisiana	Unknown
	Cetaceans	California	Unknown
	Manatees	Florida	Biotoxin
	Large whales	California	Human interaction
	Guadalupe fur seals	Northwest	Unknown
2008	Bottlenose dolphins	Texas	Unknown
	Common dolphins	NC-NJ	Unknown
	Bottlenose dolphin	Florida	Unknown
	Harbor porpoises	California	Ecological factor
2009	Bottlenose dolphin	Virginia	Unknown
2010	Manatees	Florida	Ecological factor
	Bottlenose dolphins	Florida	Unknown
	Cetaceans	Northern Gulf of Mexico	Unknown

Table 1. Marine mammal unusual mortality events since 1991 (continued)

2011	Manatees	Florida	Ecological factors
	Bottlenose dolphins	South Carolina	Unknown
	Pinnipeds	New England	Infectious disease
	Pinnipeds	Alaska	Unknown
2012	Bottlenose dolphins	Texas	Unknown
2013	California sea lions	California	Unknown
	Manatees	Florida	Unknown
	Bottlenose dolphins	Florida	Ecological factors
	Bottlenose dolphins	Mid-Atlantic	Infectious disease
2015	Large whales	Western Gulf of Alaska	Unknown
	Guadalupe fur seals	California	Unknown

Frances et al., 2007. Is Marine Mammal Health Deteriorating? Trends in the Global Reporting of Marine Mammal Disease. EcoHealth 4: 135-150. and Administrative records of the National Marine Fisheries Service (NMFS) Marine Mammal Health and Stranding Program

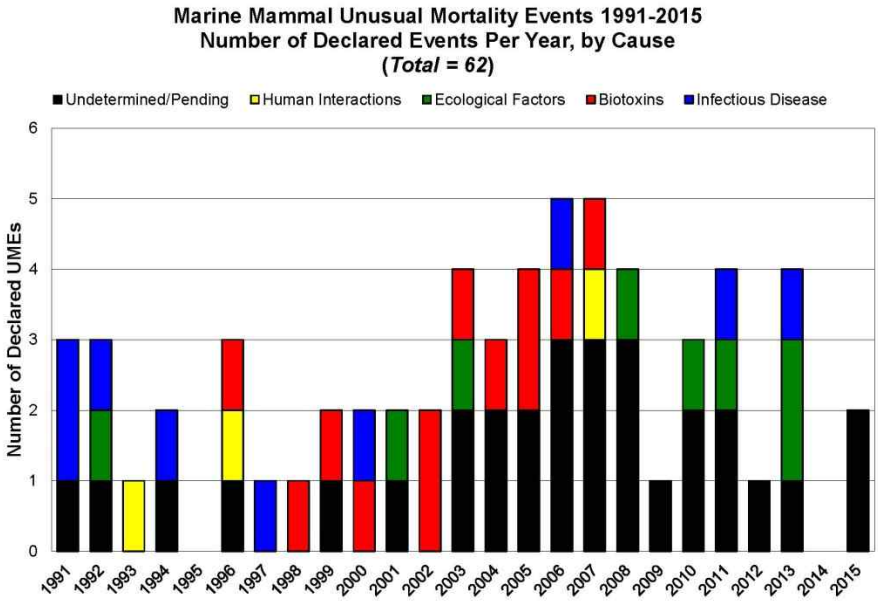

Figure 1. Number of marine mammal unusual mortality events by cause

Administrative records of the National Marine Fisheries Service (NMFS) Marine Mammal Health and Stranding Program

Marine mammal zoonoses

During the last decades, some zoonotic infectious agents have been transmitted to humans from captive marine mammals or from stranded animals. Some of these infections may possibly have little significance as zoonoses under natural circumstances, but since the contact between marine mammals and man has broadened in this manner, some reports of such infections are discussed. In addition to the known zoonotic infectious agents,

a wide range of bacteria, viruses and fungi have been isolated from marine mammals, with or without clinical signs of disease (Dierauf 1990, Higgins 2000). Some of these agents may potentially cause human infections following handling or consumption of marine mammals or marine mammal products.

An example of a commonly seen marine mammal zoonotic disease includes 'seal finger', a common skin infection reported in whalers and sealers caused by *Mycoplamsa phocicerebrale* carried in the mouth and on the skin of marine mammals (Baker et al., 1998, Hartley and Pitcher 2002). The agent causes a painful condition characterized by a severe subcutaneous tissue inflammation with, in some cases, joint involvement (Baker et al., 1998). Other reports of marine mammal workers acquiring skin diseases include: 1 case of *Mycobacterium marinum* from a bottlenose dolphin *Tursiops truncatus* (Flowers 1970); 4 cases of *Erysipelothrix rhusiopathiae* from a beached pilot whale. Infections with *M. marinum* and *E. rhusiopathiae* caused painful dermal abscesses at the site of contamination. Transmission of *Mycobacterium bovis* from a New Zealand fur seal *Arctocephalus forsteri* to an oceanarium worker has been documented (Thompson et al., 1993), with the seal trainer experiencing a tuberculous pneumonia and severe airway obstruction. Three researchers acquired leptospirosis from California sea lion *Zalophus californianus* carcasses and experienced acute nephritis and clinical signs consistent with acute renal failure (Baker et al., 1998). One laboratory

worker developed brucellosis after handling tissues from an infected seal (Brew et al., 1999). *Staphylococcus aureus* and *Vibrio parahemolyticus* were reported as zoonotic agents through occupational contact between marine mammals and humans (Palmer et al. 1991, Cowan et al., 2001).

Epidemics of food-borne illnesses, such as salmonellosis, trichinellosis, and toxoplasmosis, have also been reported in the native peoples of Arctic and Australasian regions who harvest marine mammals as part of a traditional diet (Cawthorn 1997, Tryland 2000). For example, botulism Type E, characterized by symmetric flaccid paralysis, was reported in western Alaska in people who had eaten a beached whale (McLaughlin et al., 2004).

During certain recreational activities, the public may also be at risk of transmitting diseases to and contracting diseases from marine mammals. Thousands of people visit oceanaria where contact with marine mammals (or the water in which they swim) is common.

Bacterial diseases

Bacterial diseases are major causes of mortality in wild marine mammal populations. Although bacterial infections are often secondary to primary conditions, such as viruses, phyroroxins, parasitic infections or traumatic injury, certain organisms cause disese pwrrocesses in marine mammals.

Diseases, which were seldom referenced in the earlier marine mammal, have emerged as significant causes of morbidity and mortality in both wild and captive populations of marine mammals. Brucellosis, a novel infectious disease of marine mammals, was reported in various seals, porpoises, dolphins, and otters (Foster et al., 1996). These marine mammal isolates are distinct from classically recognized terrestrial species of *Brucella* and are considered novel *Brucella* species (Jahans et al., 1997; Bricker et al., 2000; Foster et al., 2007).

The incidence of bacterial disease differs among species of marine mammals depending on habitat and behavior. Bacterial species and density vary according to proximity with human populations and discharge of human sewage into nearshore water. Pinnipeds, who haul out on land and engage in physical contact, are at higher risk for bacterial infections than other marine mammls. Most of the bacterial diseases affecting marine mammals can cause disease in terrestrial mammals and human.

A variety of novle laboratory technologies, such as the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), *in situ* hybridization, sequencing, and immunohistochemistry, have enhanced ability to identify disease etiologies. Combining the laboratory-based identification of disease etiology with long-term population monitoring by field biologists is key to understiading diseases in wildlife.

Bacterial disease is thought to be one of the leading natural causes of death in

both wild and captive marine mammals. The prevalence and significance of most bacterial species isolated from many marine mammals are unknown. There are several reasons for this relative dearth of information, including the difficulty in sampling in a marine environment, sampling from carcasses where postmortem bacterial overgrowth is likely, and the probability that severely infected animals will be removed through predation and therefore not be available to be included in these type of studies.

Brucellosis

Brucella spp. are small, facultative, intracellular, Gram-negative (Quinn and Markey, 2003). In 1994, *Brucella* spp. were isolated from marine mammals for the first time coincidently in two different locations. These strains originated from common seals (*Phoca vitulina*), harbour porpoises (*Phocoena phocoena*), a common dolphin (*Delphinus delphis*) in Scotland (Ross et al., 1994), and a captive bottlenose dolphin (*Tursiops truncatus*) in the USA (Ewalt et al., 1994). Since these first reports, marine mammal *Brucella* strains have been isolated from a wide range of marine mammal species originating from different geographic regions (Foster et al., 2002, Foster et al., 2007, Dawson et al., 2008 and Maquart et al., 2009). Marine *Brucella* spp. have been classified as two species, *Brucella ceti* and *Brucella pinnipedialis*, for isolates from cetaceans and seals, respectively (Cloeckaert et al., 2001; Foster et al., 2007; Banai and Corbel, 2010). Like terrestrial isolates, *B. ceti* and

potentially *B. pinnipedialis*, are zoonotic (Cloeckaert et al., 2011). In 1999 a case of laboratory-acquired brucellosis of marine origin occurred (Brew et al., 1999). Subsequently, three cases of naturally acquired brucellosis of marine origin were reported from people having no known history of exposure to marine mammals (Sohn et al., 2003; McDonald et al., 2006).

Marine *Brucella* spp. also can cause disease in domestic animals. Experiments using marine *Brucella* of different origins and different inoculation routes caused abortion and seroconversion in cattle (Rhyan et al., 2001), infection and seroconversion without abortion in sheep, and fulminant infection in guinea pigs (Perrett et al., 2003).

The clinical presentation and transmission in marine mammals is still poorly understood, because signs are nonspecific, and isolation of *Brucella* spp. typically comes from postmortem examinations. A few cases have reported neurologic (Hernandez-Mora et al., 2008; Gonalez-Barrientos et al., 2010) and reproductive (Miller et al., 1999) signs.

Erysipelas

Erysipelothrix rhusiopathiae is a Gram-positive and facultative anaerobic bacillus. The organism most commonly affects swine and turkeys resulting in significant economic losses (Wang et al., 2010). It infects many species including bird, rodents, sheep, reptiles, ducks, horse, marine mammals, and human. Cetaceans are the marine mammals most susceptible to the disease,

and infections have been reported since the 1950s (Seibold and Neal, 1956; Simpson et al., 1958; Higgins, 2000). In cetaceans, two distinct form of diseases have been reported, an acute septicemic form and chronic dermatologic form (Siebold and Neal 1956; Kinsel et al., 1997). The septicemic variant often results in death with no clinical signs or gross lesions (Medway 1980). Necropsy findings have included serosanguinous ascetic fluid, multifocal intestinal petechial and ecchymotic hemorrhage, sloughing skin, swollen lymph nodes, and splenomegaly. *E. rhusiopahtiae* can be isolated from lymph nodes, heart blood, kidney, and liver (Meway, 1980). Histopathologic examination reveals non-specific multifocal necrosis and inflammation of various organs, intracellular and extracellular Gram-positive bacillus (Kinsel et al., 1997).

Although acute septicemic disease occurs with no specific signs, and diagnosis is generally made postmortem, pathognomonic diamond-shaped skin lesions are seen in the dermatological form of the disease (Melero et al., 2011). It is characterized by dermal infarction results in sloughing of the epidermis (Simpson et al., 1958; Geraci et al., 1966). In bottlenose dolphins, colorless, raised, rhomboid-shaped skin lesions with well-defined edges along the entire body surface are seen (Melero et al., 2011). Affected animals are reluctant to move and erosions of the humeroscapular joint have been found (Medway, 1980). Prompt treatment with appropriate antibiotics is usually curative. If the animals are not treated, they may die.

E. rhusiopathiae is less frequently reported in pinnipeds (Lauckner 1985; Suer and Vedros, 1988) unlike the situation in cetaceans. However, the organism can infect pinnipeds. It was isolated from the teeth and gum of two elephant seals (*Mirounga angustirostris*) and two northern fur seals (*Callorhinus ursinus*) (Suer and Vedros, 1988). Necropsy findings included pulmonary hemorrhage, lymphoid depletion, and diffuse multifocal congestion and fibrosis. Nevertheless, its clinical significance has been questioned (Sweeney, 1974).

Leptospirosis

Leptospira species are long, spiral-shaped, motile Gram-negative bacteria that are 6-20 ㎛ long and 0.1 ㎛ in diameter with wavelength of about 0.5 ㎛. *Leptospira* spp. are globally distributed, infecting humans and a wide variety of domestic and wild mammal species, including several pinniped species (Smith et al., 1977; Gulland et al., 1996; Stamper et al., 1998; Colegrove et al., 2005).

Although the mode of transmission of the agent among pinnipeds is not fully understood, it likely involves direct spread among individuals via infected urine at rookeries (Cameron et al., 2008; Norman et al., 2008; Zuerner et al., 2009). Dierauf et al., (1985) indicated that sea lions could continue to shed leptospiras in urine for 154 days.

Leptospirosis in pinnipeds is characterized by depression, anorexia, pyrexia,

icterus, oral erosions or ulcers, dehydration, extreme thirst, polydipsia, fever, muscle tremors, vomiting, abortion and reluctance to use the rear limbs (Vedros et al., 1971; Smith et al., 1974; Dierauf et al., 1985; Gulland et al., 1996; Dunn et al., 2001).

At necropsy of affected animals, the kidneys are often swollen and hard, and the liver may be friable and swollen. There is a loss of differentiation between the medulla and cortex, gallbladders containing thick, black bile, and thick, pale yellow pericardial fluid (Dierauf et al., 1985; Gulland et al., 1996). In fur seal pups, subcapsular haemorrhages of kidney and liver were prominent (Smith et al., 1977).

Histopathological changes have included a lymphoplasmacytic tubulointerstitial nephritis, glomerulonephritis, intratubular protein casts. The large numbers of the spirochaetes can be identified with dark field microscopy or Warthin-Starry technique within the tubular epithelium and lumen (Gulland et al., 1996; Colegrove et al., 2005).

Mycobacteriosis

Mycobacterium spp. are aerobic, non-motile bacilli except for the species M. marinum, which bas been shown to be motile within macrophages. They are often found intracellularly. Several species of Mycobacterium have been isolated from mmarine mammals including *M. fortuitum, M. chelonei* (Bernardelli et al., 1990), *M. marinum* (Flowers 1970), *M. pinnipedii* (Kiers et

al., 2008; Scott et al., 2014; Wayne et al., 2014). By 1980s, the cases involved atypical, nontuberculous mycobacterial species (Boever et al., 1976; Morales et al., 1985; Gutter et al., 1987; Castro Ramos et al., 1998). Since the early 1990s, tuberculous mycobacterial infections have been diagnosed in several captive and wild pinnipeds (Cousins et al., 1993; Hunter et al., 1998; Thorel et al., 1998).

Pinnipeds infected with *M. pinnipedii* often display nonspecific clinical signs including anorexia, lethargy, and weight loss (Forshaw and Phelps, 1991; Kiers et al., 2008). Coughing is not a prominent feature. Wells et al., (1990) described a case of cutaneous mycobacteriosis in a captive harbor seal from which *M. chitae* and *M. fortuitum* were isolated. Diagnosis of tuberculosis can be made by intradermal tuberculin tests using bovine-pufiried protein derivative (PPD) or ELISA. Spread of the disease was thought to be via inhalation, because the lung was the focus of infection in both the wild and captive cases. The gregarious nature of many pinniped species would likely predispose to transmission via inhalation.

On necropsy, pinnipeds infected with *M. pinnipedii* display granulomas in some or all of the following organs: lungs, liver, spleen, kidneys, and lymph nodes (Forshaw and Phelps, 1991; Kiers et al., 2008; Kriz et al., 2011).

Histological examination of lesions associated with *M. pinnipedii* revealed a consistent pattern of well orientated spindle cell proliferation. Granulocytes infiltrated into the areas of necrosis and prominent lymphocyte accumulations

are seen (Forshaw and Phelps, 1991). Numerous acid-fast bacteria may be observed in some lesions (Lewis, 1987; Bowenkamp et al., 2001; Kiers et al., 2008).

Pasteurellosis

Pasteurella spp. are genus of nonmotile, pleomorphic, Gram-negative, facultatively anaerobic bacteria. Most members live as potential pathogens on mucosal surfaces of birds and mammals, colonizing mainly the upper respiratory tract, the lower reproductive tract, and possibly also parts of the intestinal tract (Christensen and Bisgaard, 2008). The organisms have been reported from not only diseased marine mammals, but also healthy individuals (Dunn et al., 2001; Higgins 2000). Lockwood et al. (2003) isolated *Pasteurella* spp. from the conjunctiva and wounds of stranded harbor seals (*Phoca vitulina*). *Pasteurella canis* were isolated from three female California sea lions, and *Pasteurella stomatis* from grey seals and harbor seals (Hansen et al., 2012). *P. canis* and *P. stomatis* are frequently isolated from the oral cavity of other members of the *Carnivora* (Mutters et al., 1985; Talan et al., 1999).

P. multocida has been cultured from cetaceans (Duun et al., 2001). Medway et al. (1973) isolated *Pasteurella* spp. from the lungs of a bottlenose dolphin with acute hemorrhageic bronchopneumonia. Kennedy-Stoskopf et al. (1986) described a *P. multocida*-associated pericarditis and septicemia in a California

sea lion.

Pasteurellosis most often manifests as an acute or peracute septicemia. Death often occurs either without clinical signs or only a few hours after the development of anorexia or other behavioral signs, such as lethargy, decreased swimming, or failure to interact normally with pool mates. The majority of case reports have had very few gross lesions, which is consistent with their peracute nature.

The necropsy findings included diffusely edematous lungs and a diffuse acute lymphadenitis, fat necrosis in the blubber near the cervical esophagus, cervical swelling, epcardial and pericardial petechiation. Histopathological findings include splenitis, hepatitis, interstitial and bronchial pneumonia, myocarditis, and nephritis. The agent was isolated from several organs (Dunn et al., 2001).

The long-term immunity has been provided after administration of primary and booster vaccinations using polysaccharide vaccine developed from isolate (Vedros, 1982). Sensitivity test and application of appropriate antibiotics show that P. multocida is easily controlled with antibiotics. However, the peracute nature of the disease has far resulted in a situation where treatment has been of no value once the disease is clinically obvious (Dunn et al., 2001).

Nocardiosis

Nocardia spp. are weakly staining Gram-positive, catalase-positive, rod-

shaped, aerobic bacteria. *Nocardia* is found worldwide in soil, vegetable matter, and water (Ryan and Ray, 2004). Some species are nonpathogenic, while others are responsible for nocardiosis. Pathogenic *Nocardia* spp. include *N. asteroides*, *N. brasiliensis*, *N. otitidiscaviarum*, and *N. transvalensis* (Williams et al., 1983). In mammals, 6 basic forms can be distinguished: pulmonary; systemic; central nervous system; extrapulmonary; cutaneous, subcutaneous, or lymphocutaneous; and actinomycetoma (St. Legar et al., 2009).

The current published reports of marine mammal nocardiosis show high mortality like in humans and dogs (Davis et al., 1977; Macneill et al., 1978; Martineau et al., 1988. Pier et al., 1970; Sweeny et al., 1976; Vemireddi et al., 2007). Current literature suggests that the systemic form is the most common presentation in cetaceans (Macneill et al., 1978; Martineau et al., 1988). There is one report of pulmonary nocardiosis in a Pacific bottlenose dolphin (*Tursiops aduncus*) (Pier et al., 1970).

Pulmonary or extrapulmonary nocardiosis are the forms of the disease most often noted in cetaceans. *Nocardia* spp. may easily become airborne on dust particles and inhaled, since the organisms are ubiquitous in environments (Dunn et al., 2001). Because cetaceans appear to be vulnerable to pneumonia, any environmental activity that could increase exposure to soil-borne particle should be considered as possible causes of infection (Dunn et al., 2001).

Only 1 case of nocardiosis in a pinniped has been reported by the mid-2000s.

However, in 2009, St. Leger et al. reported naturally occurring nocardiosis of hooded seals and leopard seal. The necropsy finding included pyothorax, abscesses of lung, thoracic lymph node, and brain, pleuritic, pyogranulomas in skin, liver, spleen. The pathogenic species were identified with *N. asteroids*, *N. otitisdiscaviarum*, *N. farcinica*, and *N. brasiliensis* (St Leger et al., 2009).

Mycoplasmosis

Mycoplasmas species are the smallest bacterial cells yet discovered, and lack a cell wall around their cell membrane. *Mycoplasma* spp. have commonly been isolated from mucosal surfaces of healthy animals and may be inoculated into the skin via bite wounds, resulting in subcutaneous abscesses and secondary infection. Also, *Mycoplasma* spp. have been associated with viral-induced marine mammal mass mortality events (Madoff et al., 1982; Giebel et al., 1991; Ruhnke and Madoff, 1992). In pinnipeds, five species are known: *Mycolpamsa phocidae, Mycoplasma phocirhinis, Mycoplasma phocicerebrale, Mycoplasma zalophi and Mycoplasma haemozalophi* (Madoff et al., 1982; Kirchhoff et al., 1989; Giebel et al., 1991; Haulena et al., 2006; Volokhov et al., 2011). *M. phocidae* was cultured from the respiratory tract and heart of harbour seals during a respiratory epizootic along the New England seaboard from 1979 to 1980 (Madoff et al., 1982). *M. phocicerebrale* and *M. phocirhinis* were cultured from seals during a mass mortality event that occurred in the Baltic and North Sea (Kirchhoff et al., 1989; Giebel et al.,

1991). *M. zalophi* was repeatedly isolated from California sea lions (*Zalophus californianus*) undergoing rehabilitation from 1999 to 2001 (Haulena et al., 2006). Also, *Mycoplasma zalophi* were proposed as the sole etiologic agents of abortion in a colony of Australian fur seals (Lynch et al., 2011). Aborted fetuses cultured positive for *Mycoplasma* spp. and showed pulmonary lesions typical of *Mycoplasma* infection. The authors speculated that either ascending genital infection or sepsis led to fetal exposure and abortion as seen in humans and cattle. However, their role as a primary pathogen remains unclear as other agents are typically isolated simultaneously.

A recent study isolated *M. phocicerebrale* and at least two novel *Mycoplasma* spp. from cetacean carcasses that washed up off the coast of Scotland over a 12-year period (Foster et al., 2011).

In marine mammals, *Mycoplasma* spp. are often associated with respiratory disease signs (Giebel et al., 1991; Ruhnke and Madoff, 1992) and have been associated with significant stranding or mortality events (Giebel et al., 1991; Ruhnke and Madoff, 1992; Foster et al., 2011). Mycoplasma zalophi is associated with pneumonia and polyarthritis in California sea lions in rehabilitation (Haulena et al., 2006).

Few gross lesions are associated with *Mycoplasma* infections in marine mammals. Histopathologic findings typically include a pleuritis, interstitial pneumonia or bronchopneumonia, lymphadenitis, subdermal abcessation and septic polyarthritis (Haulena et al., 2006; Foster et al., 2011).

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Chapter 1

Dietzia cinnamea and *Clostridium tertium* from a finless porpoise (*Neophocaena asiaeorientalis*) in Republic of Korea

Abstract

Bacterial examination of the tissues of a male finless porpoise (*Neophocaena asiaeorientalis*) found dead in February 2010 in Tongyeong, South Korea, was performed, and *Dietzia cinnamea* and *Clostridium tertium* were isolated. To our best knowledge, this is the first reported case of *D. cinnamea* and *C. tertium* in a marine mammal in Republic of Korea.

Keywords: *Clostridium tertium*, *Dietzia cinnamea*, finless porpoise, *Neophocaena asiaeorientalis*, Republic of Korea

Introduction

The finless porpoise is a small porpoise that lacks a dorsal fin. Although the taxonomy of finless porpoise is controversial, the currently accepted classification is of two separate species, *Neophocaena phocaenoides* and *Neophocaena asiaeorientalis*, which are characterized by differences in the skull morphology (Li et al., 2013). *N. asiaeorientalis* has a narrow ridge on its back (Li et al., 2013) this subspecies inhabits the northern part of the East China Sea, the Yellow Sea, and the waters of Korea and Japan. In Korea, finless porpoises are found mainly in the Yellow Sea and around the islands in the South and East Seas. According to a sighting survey conducted from 2004 to 2005 off the west coast of Korea, the estimated abundance of finless porpoise was approximately 36,000 (Park et al., 2014). However, a mass mortality of 249 finless porpoises occurred in the Saemangeum Dyke in 2011, and the number of carcasses found at the shore or riverside has increased recently (Park et al., 2014).

Although the exact cause of this decrease is not fully understood, the possibility of infection of pathogenic organisms might be worth consideration. However, to date, there have been very limited reports about bacteria associated with finless porpoises (McLaughlin et al., 2012). Here, we present a unique case of isolation and identification of *Dietzia cinnamea* and *Clostridium tertium* from a stranded finless porpoise.

Materials and methods

On February 22, 2010, a finless porpoise was found dead on the seashore in Tongyeong in South Korea. The animal was transported to the Cetacean Research Institute, and stored in a freezer until a necropsy was performed. Samples of the liver, lung, stomach, intestine, pancreas, kidney, spleen, testis, epididymis, and lymph nodes were taken for bacteriological evaluation. The tissue samples were homogenized and cultured on sheep's blood agar, tryptic-soy agar and tryptic-soy broth. The plates and broth tubes were incubated at 37°C in aerobic and anaerobic conditions for more than 7 days until some bacteria could be identified with the naked eye. Bacterial identification was performed using the Vitek 2 identification system (BioMerieux), 16S rRNA gene sequencing, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. Antimicrobial susceptibility of the isolates was determined by E-tests (BioMerieux) against antibiotics such as penicillin, amoxicillin-clavulanic acid, ciprofloxacin, ofloxacin, gentamicin, and ceftriaxone.

Results

The finless porpoise was determined to be male, approximately 182 cm in length, and his age was estimated to be at least 5 years old, based on body length and sexual maturity. The finless porpoise had a normal external appearance, except for several minor cuts. Gross examination of the internal organs revealed no other lesions.

After 48 h of incubation of tissue samples, semitransparent, small-size colony was isolated on the blood agar plate streaked with intestine tissue under anaerobic conditions. A similar colony also grew under aerobic conditions. Furthermore, after 3 days of incubation in aerobic conditions, a yellow colony was found to be growing on the blood agar plate streaked with lung tissue. Gram staining revealed gram variable and positive rod from colonies of intestine and lung tissue, respectively. Bacterial identification was performed using the Vitek 2 identification system (BioMerieux), 16S rRNA gene sequencing, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis.

None of the isolates were identifiable using the Vitek 2 identification system. Results of the 16S rRNA gene sequencing revealed that the isolate from the lung tissue showed the highest sequence similarity (99%) with *D. cinnamea* (strain DSM 44904). The same result was obtained with MALDI-TOF MS. Further, the 16S rRNA sequences of a colony from the intestinal tissue were

98% identical with *C. tertium* (strain JCM 3813). This result was also consistent with that obtained using MALDI-TOF MS.

Antimicrobial susceptibility of the isolates was determined by E-tests (BioMerieux) against antibiotics such as penicillin, amoxicillin-clavulanic acid, ciprofloxacin, ofloxacin, gentamicin, and ceftriaxone. The minimal inhibitory concentration (MIC) values of these drugs were determined (Table 1-1). The non-species-related breakpoints used for the antimicrobials were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). *D. cinnamea* was susceptible to all antimicrobial agents used in this study, while *C. tertium* was susceptible only to amoxicillin-clavulanic acid.

Table 1-1. Minimum inhibitory concentrations (MICs) of the isolates

Antimicrobial agent	MIC (μg/ml)	
	D. cinnamea	*C. tertium*
Penicillin	0.094	1.0
Ammoxicillin-clavulanic acid	0.094	0.75
Ciprofloxacin	0.094	1.0
Ofloxacin	0.25	2.0
Gentamicin	0.032	>256
Ceftriaxone	0.75	>32

Discussion

D. cinnamea is an aerobic, nonsporing, non-acid fast, gram-positive bacterium that was first isolated from the perianal region of a bone marrow transplant patient in 2006 (Yassin et al., 2006). Its morphology ranges from rod- to coccoid-shaped, and forms non-mucoid and yellow-pigmented colonies. Most commercial identification systems usually misidentify *Dietzia* spp. as *Rhodococcus* spp.; therefore, additional tests such as 16S rRNA and *gyrB* gene sequencing analyses are needed to differentiate between them (Hirvonen et al., 2012; Niwa et al., 2012). In the present study, *D. cinnamea* could be identified using 16S rRNA gene sequencing and MALDI-TOF MS. MALDI-TOF MS is known as a powerful tool for the identification of bacteria, and has been used in clinical laboratories (Salvador et al., 2013). It can be applied to the identification of bacteria that are difficult to identify using conventional methods. In addition, it is easy to perform, and provides rapid and reliable results.

Only a few *Dietzia* species have been implicated as agents of human diseases (Koerner et al., 2008). In addition, there have been few reports about clinical isolates since the first isolation of *D. cinnamea*, and their pathogenesis is still not well known (Hirvonen et al., 2012; Koerner et al., 2008). To our knowledge, to date, there has been no report about isolation of *D. cinnamea* from animals, and this is believed to be the first report on the identification of

D. cinnamea from a finless porpoise. In this study, *D. cinnamea* isolate was found to be generally susceptible to most antimicrobial agents, similar to in other studies (Hirvonen et al., 2012; Niwa et al., 2012). It was highly susceptible to gentamicin with a minimal inhibitory concentration of 0.032 μg/ml.

C. tertium is a non-toxin producing, predominantly anaerobic and aerotolerant gram-positive bacillus first isolated from war wounds in 1917 (Henry, 1917). *C. tertium* is distributed in the soil and gastrointestinal tracts of animals and humans. There are several reported cases of *C. tertium* infection in human (Miller et al., 2001; Salvador et al., 2013; Vanderhofstadt et al., 2010). Moreover, *C. tertium* is the second most frequently isolated species out of *Clostridium* spp. causing clostridial bacteremia (Salvador et al., 2013). Many *Clostridium* species, including *C. perfringens*, *C. novyi*, and *C. chauvoei,* are found in various tissues of marine mammals (Buck et al., 1987); however, the identification of *C. tertium* is rare, not only in terrestrial mammals but also in marine mammals.

In human cases, *C. tertium* has been identified in blood of neutropenic patients as an important cause of sepsis (Miller et al., 2001; Salvador et al., 2013; Vanderhofstadt et al., 2010). In the present case, we did not check the blood of the finless porpoise, and could hence not determine the presence of bacteremia. However, similar to in humans, the possibility of *C. tertium* bacteremia should be considered in marine mammals. In human cases, most

patients with *C. tertium* infection reportedly had severe underlying diseases (Fujitani et al., 2007; Miller et al., 2001; Vanderhofstadt et al., 2010; You et al., 2015). Although *C. tertium* has been regarded as nonpathogenic, and found in both animal and human gastrointestinal tracts and in the soil, as shown in many studies, it can indeed cause clinical diseases (Fujitani et al., 2007; Salvador et al., 2013; Šeol et al., 2006; Silvera et al., 2003; You et al., 2015). One study showed that *C. tertium* caused clinical disease such as fever in humans (Miller et al., 2001), while *C. tertium* isolated from a dolphin was determined as the cause of abscessation, osteomyelitis, and death (Šeol et al., 2006), and that isolated from calves and a pig was found to be associated with enteritis (Silvera et al., 2003). Moreover, Silvera et al. (2003) showed experimental reproduction of diarrhea in cattle. However, the virulence and pathogenic mechanism of *C. tertium* have not yet been determined.

In agreement with previous studies (Fujitani et al., 2007; Salvador et al., 2013; Vanderhofstadt et al., 2010), *C. tertium* isolate was found to be resistant to penicillin and sensitive to amoxicillin-clavulanic acid in the present study. However, You et al. (2015) reported that clinical isolate from blood in a patient with glyphosate ingestion was susceptible to penicillin.

Finally, this study did not try to demonstrate the two isolates, *D. cinnamea* and *C. tertium*, in the blood or tissues of the finless porpoise. Some authors have expressed doubts about the clinical importance of the identification of *D. cinnamea* and *C. tertium*. However, the cause of the mass stranding of finless

porpoises in South Korea has not yet revealed, and there are currently no other clues to demonstrate the cause of death in these animals. Therefore, the possibility of these strains as pathogens in finless porpoises needs to be considered.

In conclusion, this is the first case of isolation of *D. cinnamea* and *C. tertium* from a finless porpoise in South Korea. However, we could not determine whether these organisms resulted in any clinical symptoms or if their infection contributed to the death of the finless porpoise. Although there are some case reports of human and animal infections with these bacteria, the presence of direct transmission between humans and animals is not clear. Therefore, the possibility of zoonotic infection cannot be ruled out, and further studies are needed to determine the impact of *D. cinnamea* and *C. tertium* on the health of marine mammals.

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Chapter 2

Streptococcus halichoeri and *Streptococcus phocae* from a Steller Sea Lion (*Eumetopias jubatus*) in Republic of Korea

Abstract

Streptococcus species are emerging potential pathogens in marine mammals. This study presents the first results of the isolation and identification of *Streptococcus halichoeri* and *Streptococcus phocae* in a Steller sea lion, *Eumetopias jubatus*, in Republic of Korea.

Keywords*: Streptococcus halichoeri, Streptococcus phocae,* steller sea lion, *Eumetopias jubatus*, Republic of Korea

Introduction

In marine mammals, streptococci have been implicated in debilitating disease processes such as pneumoniae, septicemia, and opportunistic infections (Imai et al., 2009; Johnson et al., 2006). These marine mammal isolates have been identified as *Streptococcus phocae*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, *Streptococcus marimammalium*, *Streptococcus halichoeri*, *Streptococcus iniae*, *Streptococcus canis*, and *Streptococcus zooepidermicus* (Pier and Madin, 1976; Skaar et al., 1994; Swenshon et al., 1998; Vossen et al., 2004; Lawson et al., 2004; Lawson et al., 2005; Johnson et al., 2006; Kuiken et al., 2006). In particular, *Streptococcus phocae* is known as an important pathogen causing pneumonia or respiratory infection in pinnipeds (Skaar et al., 1994; Vossen et al., 2004). In addition to, Streptococcus halichoeri, first reported in 2004, was isolated from gray seals, and found in humans.

Here, we present a unique case of isolation and identification of *Streptococcus halichoeri* and *Streptococcus phocae* from a Steller sea lion, *Eumetopias jubatus*, in Republic of Korea.

Materials and Methods

In the winter of 2012, an adult female Steller sea lion appeared at Biyang-do, a small island near Jeju-do in South Korea. However, it soon disappeared and was found dead after about 2 weeks in the vicinity of the original observation (Figure 2-1). The female Steller sea lion's body length was greater than 2.8 m and her body weight was approximately 300 kg. The animal's age was estimated to be 6 years old based on body size, weight and dental measurements.

Postmortem examination was performed. Samples of the lung, liver, stomach, intestine, kidney, spleen, uterus, ovary and lymph nodes were taken for bacteriological and pathological evaluation. Each tissue was fixed with formalin for microscopic examination. The tissue samples were homogenized and cultured on sheep's blood agar, tryptic-soy agar and tryptic-soy broth. The plates were incubated at 37℃ in aerobic conditions and a 5% CO_2-enriched atmosphere for more than 7 days until some bacteria could be identified with the naked eye. Bacterial identification was performed using the Vitek 2 identification system (BioMerieux), 16S rRNA gene sequencing, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis.

Figure 2-1. Female Steller sea lion found at Biyang-do, a small island near Jeju-do in Republic of Korea

Results

Although there was some skin slippage, she was in moderate post mortem condition. The internal organs were severely decayed because the necropsy was performed long after death. There was not any indication of pregnancy. Unfortunately, the pathological examination could not yield specific findings owing to the poor condition of the decayed tissue samples.

After 24 h of incubation of tissue samples, tiny, nonhemolytic and hemolytic colonies grew on the blood agar plates streaked with kidney and liver tissues, respectively. The Vitek 2 identification system (bioMerieux), 16S rRNA gene analysis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis were performed to identify the isolates. Two isolates were identified as gram-positive cocci that were growing in chains.

None of the isolates were identifiable using the Vitek 2 identification system. The results of 16S rRNA gene sequencing revealed that the isolate from the kidney showed the highest sequence similarity of 99% with *Streptococcus halichoeri* isolated from grey seals in 2004 (accession number AJ606046). This result was consistent with that obtained using MALDI-TOF MS. This isolate was classified as Lancefield group B, according to the Lancefield streptococcal grouping test (Oxoid Ltd., Basingstoke, England).

The 16S rRNA gene analysis showed that the isolate from the liver displayed

sequence similarity greater than 98% with *Streptococcus phocae* isolated from salmon (accession number AJ621053). The same result was obtained with MALDI-TOF MS. The Lancefield grouping test showed that this isolate belongings to Lancefield group G.

Discussion

Steller sea lions (*Eumetopias jubatus*) are usually distributed from the Kuril Islands and the Sea of Okhotsk to the Gulf of Alaska in the north, and south to Año Nuevo Island in central California. In the summer, Steller sea lions occasionally move southward and have been found on the coasts of Korea and China. Although they are very occasionally spotted in the East Sea in the winter, this case represents the first appearance of a Steller sea lion in Jeju Island in South Korea.

The population of Steller sea lions has decreased since the 1970s. Although the cause of this decline is not clearly known, infectious disease may play a role in threatening this population. Few studies on the pathogenic bacteria of Steller sea lions have been performed; however, bacteria including *Pasteurella* spp., *Streptococcus* spp., *Mannheimia* spp., *Escherichia coli*, and *Salmonella* spp. were isolated from the rectal and oral cavities of Steller sea lions in Alaska (Carrasco et al., 2011).

Streptococcus spp. have been frequently isolated from different kinds of aquatic animals and are associated with diseases such as pneumonia and septicemia (Skaar et al., 1994). *S. phocae* was first isolated from harbor seals in 1994 (Skaar et al., 1994). Subsequently, this species was detected in grey seals (Vossen et al., 2004), spotted seals (Hueffer et al., 2011), fur seals (Henton et al., 1999), sea otters (Imai et al., 2009), Atlantic salmon (Gibello et

al., 2005; Romalde et al., 2007) and Indian white shrimp (Satish and Arul, 2009).

In seals, *S. phocae* has been isolated from several organs, including the lung, liver, and ovary, implicating a role for pneumonia, septicemia, and pyometra (Skaar et al., 1994; Vossen et al., 2004; Hueffer et al., 2011). Furthermore, in salmon, this species was found to cause exophthalmia, ventral petechial hemorrhages, deep ulcer with muscle liquefaction, and pericarditis, resulting in economic losses to the fisheries industry (Romalde et al., 2007). In the present case, the *S. phocae* infection could not be directly associated with disease. However, considering the association of *S. phocae* with urogenital carcinoma in California sea lions (Johnson et al., 2006), this species should be considered as a potential disease-causing pathogen in Steller sea lions.

In 2004, a new bacterium, *S. halichoeri*, was isolated from grey seals (Lawson et al., 2004). To date, *S. halichoeri* has only been isolated from grey seals (Lawson et al., 2004) and a Chinese man (Rui and Douglas, 2014), and thus this is believed to be the first report on the identification of *S. halichoeri* from Steller sea lions. In the human case, the bacterium could be identified by MALDI-TOF MS (Rui and Douglas, 2014). However, we were also able to identify the bacterium using 16S rRNA gene sequencing in addition to MALDI-TOF MS. Although MALDI-TOF MS is a good method for accurate and rapid identification of bacteria, the equipment is very expensive, making its laboratory use somewhat limited. Therefore, the development and

improvement of diagnostic methods might be needed to identify this species.

This is the first case of the simultaneous isolation of *S. phocae* and *S. halichoeri* from a Steller sea lion in South Korea. However, we could not prove that these organisms resulted in any clinical symptoms or if their infection contributed to the death of the Steller sea lion. There has been no report of human infection with *S. phocae* to date, and the direct transmission of *S. halichoeri* between humans and marine mammals is unclear. However, the possibility of zoonotic infection cannot be ruled out. Therefore, further studies are needed to determine the impact of *S. halichoeri* and *S. phocae* to the health of marine mammals.

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Chapter 3

Bacteria isolated from Long-beaked Common Dolphins (*Delphinus capensis*) in Republic of Korea

Abstract

Marine mammals are often cited as sentinels of ocean health yet accessible, synthesized data on their health changes that could effectibely warn of ocean health changes are rear. The objective of this study was to investigate bacterial disease of cetaceans in Republic of Korea. A total of 44 long-beaked common dolphins (*Delphinus capensis*) were taken in the South and East Seas coast. From tissue samples, bacteria were isolated, and the Vitek 2 identification system (bioMerieux), 16S rRNA gene analysis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis were performed to identify the isolates. Twenty-one spcies of bacteria were cultured from tissue samples of long-beaked common dolphins. Of the total 21 species, several species are known as bacterial pathogen to marine mammals humans.

Keywords: bacteria, *Delphinus capensis,* long-beaked common dolphin, Republic of Korea

Introduction

Bacterial disease is thought to be one of the leading natural causes of death in both wild and captive marine mammals. The prevalence and significance of most bacterial species isolated from many marine mammals are unknown. There are several reasons for this relative dearth of information, including the difficulty in sampling in a marine environment, sampling from carcasses where postmortem bacterial overgrowth is likely, and the probability that severely infected animals will be removed through predation and therefore not be available to be included in these type of studies (Dunn et al. 2001). Compared with other marine mammals, there is scarcity of microbiological data for the long-beaked common dolphins (*Delphinus capensis*) (Bossart 2001). Long-beaked common dolphins are commonly found off the coasts of California and Mexico, South America (Peru, Chile, Venezuela, Brazil, and Argentina), West Africa, South Africa, Madagascar, the Arabian Peninsula, India, Indonesia, China, Korea, and southern Japan.

One report described miscellaneous lesions of the head, skull, teeth, trunk, appendages, skin and genital tract observed in 120 of 930 long-beaked common dolphins taken in fisheries off Peru between 1985 and 2000. The majority of traumas encountered was diagnosed as caused by violent, fisheries-related interactions, and the skin in 20.4% of specimens showed healed scars from such interactions (Van Bressem et al. 2006).

The present study described the bacterial isolates found in 44 long-beaked common dolphins taken in the South and East Seas coast of Republic of Korea. We conducted antibody test to *Brucella* spp. recognized as important emerging diseases in cetaceans.

Materials and methods

A total of 44 long-beaked common dolphins were taken in the East Sea coast (Figure 3-1). They were bycaught in stow nets on anchors from February 2012 to August 2013. The dolphins were placed in freezer after recovery, and examined and necropsied after a long time.

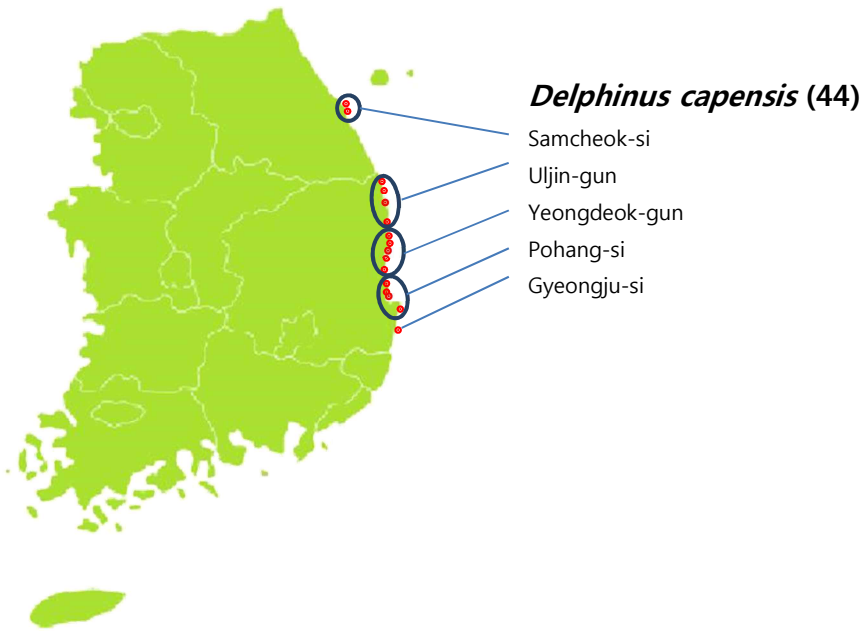

Figure 3-1. Location of incidental catch of long-beaked common dolphins.

Total body length (TBL) was measured from the tip of the beak to the notch in fluk in the unit 0.1 cm. Sexual maturity of females was determined by the

presence of one or more corpora (Perrin and Donovan, 1984). Immature females were defined as those with no corpora on ovaries, mature ones had at least one corpus on ovaries. Reproductive status of males wea assessed based on the weight of testis.

Gross examination involved visual inspection and collection of tissues from the brain, pituitary gland, heart, lungs, liver, kidney, bladder, spleen, lymph nodes, skeletal muscle, esophagus, stomach, pancreas, intestines, testis, uterus, and ovary for bacteriological evaluation. The tissue samples were homogenized and cultured on sheep's blood agar, tryptic-soy agar and tryptic-soy broth. The plates and broth tubes were incubated at 37°C in aerobic and anaerobic conditions for more than 7 days until some bacteria could be identified with the naked eye. All other collected tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 to 7 mm, stained with hematoxylin and eosin (HE), and examined microscopically. Testing for antibodies to *Brucella* spp. was conducted using rose bengal test, tube agglutination test and ELISA kit (SvanovirH, Svanova Biotech, Uppsala, Sweden).

The Vitek 2 identification system (bioMerieux), 16S rRNA gene analysis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis were performed to identify the isolates.

Results

Of 44 long-beaked common dolphins, 21 individuals were female, and 10 females were mature. Among 23 males, 8 dolpins were mature (Table 3-1). The average TBLs of the males were longer than female, and the mature dolphins had longer TBLs than immature ones.

The long-beaked common dolphis had a normal external appearance, except for several minor cuts. Gross and microscopic examination of the internal organs revealed no other lesions (Figure 3-2). There was no serologic evidence of exposure to *Brucella* species in any of the dolphin samples.

Twenty-one spcies of bacteria were cultured from tissue samples of long-beaked common dolphins (Table 3-2). Of the total 21 bacteria, some species are known as bacterial pathogen to marine mammals.

Table 3-1. Biological information of long-beaked common dolphins (No. 1-44)

No	Sample ID	Sex	TBL (cm)	Reproductive status
1	CRI 00015	Female	241.9	Mature
2	CRI 00017	Female	211.3	Immature
3	CRI 00018	Female	229.2	Mature
4	CRI 00019	Male	194.5	Immature
5	CRI 00020	Male	243.0	Mature
6	CRI 00021	Male	240.6	Mature
7	CRI 00022	Male	210.0	Immature
8	CRI 00023	Female	216.0	Immature
9	CRI 00024	Female	220.4	Mature
10	CRI 00025	Male	264.1	Mature
11	CRI 00026	Male	242.5	Mature
12	CRI 00027	Female	175.0	Immature
13	CRI 00028	Female	231.9	Mature
14	CRI 00029	Male	208.0	Immature
15	CRI 00030	Male	233.0	Mature
16	CRI 00031	Male	232.7	Mature
17	CRI 00032	Female	233.0	Mature
18	CRI 00033	Female	230.0	Mature
19	CRI 00034	Male	217.5	Immature
20	CRI 00035	Female	167.0	Immature
21	CRI 00038	Male	242.0	Mature
22	CRI 00039	Male	183.1	Immature
23	CRI 00040	Female	220.5	Mature
24	CRI 00041	Female	219.8	Mature
25	CRI 00042	Female	194.6	Immature

Table 3-1. Biological information of long-beaked common dolphins (No. 1-44) (continued)

No	Sample ID	Sex	TBL (cm)	Reproductive status
26	CRI 00043	Male	219.7	Immature
27	CRI 00044	Male	186.6	Immature
28	CRI 00045	Female	231.5	Mature
29	CRI 00046	Male	230.6	Immature
30	CRI 00047	Male	203.2	Immature
31	CRI 00096	Female	201.2	Immature
32	CRI 00097	Female	217.2	Immature
33	CRI 0064	Male	193.4	Immature
34	CRI 0065	Male	223.3	Immature
35	CRI 0066	Male	229.2	Immature
36	CRI 0067	Female	178.9	Immature
37	CRI 0068	Male	236.7	Mature
38	CRI 0069	Male	202.3	Immature
39	CRI 0306	Male	214.3	Immature
40	CRI 0307	Female	183.4	Immature
41	CRI 0166	Female	194.9	Immature
42	CRI 0179	Male	224.6	Immature
43	CRI 0196	Female	208.9	Immature
44	CRI 0199	Female	234.7	Mature

Table 3-2. Bacteria isolated from 44 long-beaked common dolphins (*Delphinus capensis*)

Bacteria	Tissues	Characteristics
Vagococcus fluvialis	Lymph node, stomach, intestine	Gram(+), motile, isolated from human and domestic animals
Vibrio sp.	Blood	Gram(-), isolated from dolphins
Actinobacillus delphinicola	Brain, lung, kidney	Gram(-), isolated from dolphins
Wautersiella falsenii	Ovary	Gran(-), isolated from clinical specimens in humans
Stenotrophomonas maltophilia	Liver, kidney, vagina	Gram(-), found in water, soil & plant, nosocomial infection
Pseudomonas spp.	Lymph node, lung, stomach	Gram(-), isolated from marine sponge, nosocomial infection
Empedobacter brevis	Uterus, ovary	Gram(-), isolated from dog & human
Photobacterium damselae subsp. *damseale*	Lung, liver	Gram(-), cause disease in fish, dolphins, fatal infection in human
Acinetobacter spp.	Brain, liver, intestine	Gram(-), important soil organism
Myroides sp.	Heart, lung	Gram(-), found in soil & fresh water, opportunity infection
Aerococcus viridans	Spleen	Gram(+), cause disease in lobster and human

Table 3-2. Bacteria isolated from 44 long-beaked common dolphins (*Delphinus capensis*) (continued)

Bacteria	Tissues	Characteristics
Lactococcus garvieae	Uterus, kidney	Gram(+), commonly used in fermented dairy products, isolated from a bottlenose dolphin
Carnobacterium divergens	Heart, uterus, testis, spleen, liver	Gram(+), cause disease in fish, bacteremia in hunam
Kurthia zopfii	Intestine	Gram(+), found in soil, feces & water
Staphylococcus equorum	Kidney	Gram(+), isolated from skin of horses and human, sausage and cheese
Staphylococcus epidermidis	Lymph node	isolated from Beluga whale
Staphylococcus cohnii	Lung	Nosocomial infection
Enterococcus spp.	Intestine	Gram(+), found in intestine & marine environment
Lactobacillus sakei	Adrenal gl., spleen, thyroid	Gram(+), found in fish and fresh meat
Lysinibacillus sphaericus	Spleen	Gram(+), found in soil, produce insecticidal toxin
Macrococcus caseolyticus	Liver	Gram(+), isolated from animal skin and food

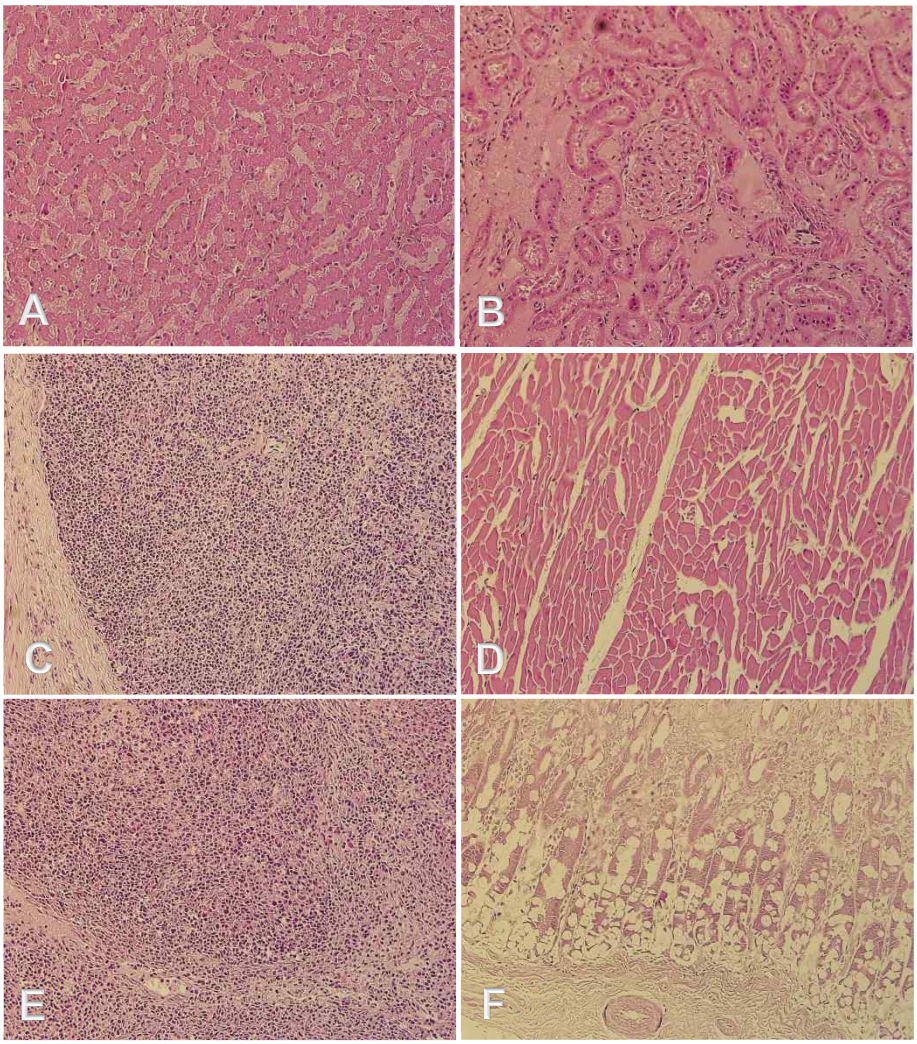

Figure 3-2. Hematoxylin and eosin stained sections from long-beaked common dolphins. (A) liver, (B) kidney, (C) lymph node, (D) myocardium, (E) pancreas, (F) intestine. 200x

Discussion

The significance of the majority of isolates cultured from these marine mammals is unclear. However, this paper provides the first documented list of isolates from long-beaked common dolphins of Republic of Korea.

Some of the isolates may have no association with diseases and are likely to represent secondary or opportunistic infections, but all may cause pathology under the right environmental and health status conditions (Thornton et al. 1998). In addition to, bacteria such as *Enterococcus* spp., *Aerococcus* spp., have been reported as pathogens in humans with severe underlying conditions or immunosuppression.

Twenty-one different bacterial species were isolated from dolphins in this study. Among these species, some bacteria were reported in marine mammals and humans.

Vibrio species can be carried by numerous marine animals, such as crabs or prawns, and several species are pathogens. *V. alginolyticus* was isolated from the blood and other organs of an Atlantic white-sided dolphin (*Lagenorynchus acutus*) (Tangredi and Medway, 1980), and *V. cholera, V. parahemolyticus* from harbor seals (Greig et al., 2014).

Actinobacillus delphinicola was reported for the first time in 1996. Isolates were recovered from cetaceans around the Scottish coastline; until now, this bacterium has not been found in other marine mammals (Higgins, 2000). *A.*

delphinicola has been isolated from various tissues; the stomach and intestinal contents of harbor porpoises (*Phocoena phocoena*); the lungs, gastric and mandibular lymph nodes, and intestinal content of a striped dolphin (*Stenella coeruleoalba*); and the lungs of a Sowerby's beaked whale (*Mesoploden bidens*) (Foster et al, 1996).

Lactococcus garvieae has been isolated from clinical specimens of human skin, blood, and urine and is also a known etiological agent of mastitis in cows (Collins et al., 1983; Elliott et al., 1991; Vela et al., 2000). This species is an established pathogen of fish, causing a variety of clinical signs and significant mortalities in fish including rainbow trout (Ravelo et al., 2001; Chang et al., 2002), yellowtail (Zlotkin et al., 1998), and grey mullet (Chen et al., 2002). The organism was also isolated from a freshly dead bottlenose dolphin (*Tursiops truncatus*) from Kuwait Bay (Evans et al. 2006). *L. garvieae* should therefore be considered among the list of potential marine mammal pathogens.

Acinetobacter species are also isolated from marine mammals. *A. lwoffii* was cultured from blood of bottlenose dolphins (Venn-Watson et al., 2008). This was strongly suspected to be the etiology of abnormal tissue or clinical illness. However, this was not associated with mortality or the cause of mortality.

Enterococcus species were commonly isolated from several species of pinnipeds. It was cultured from superficial tissue infections, lung with histologic characteristics of pneumonia, liver from septicemic animals, and

brain with histologic evidence of encephalitis or meningitis (Thornton et al. 1998). In humans, immunosuppressed individuals are most at risk for hematogenous meningitis due to secondary enterococcal infections outside the central nervous system (Pintado et al., 2003).

The presence of *Brucella* antibodies in marine mammals has been reported from many geographic areas and species, indicating that *Brucella* infections affect a large number of cetacean and pinniped species and have a worldwide distribution (Nielsen et al., 2001b; Tryland et al., 2005). Antibody tests including rose-bengal test, tube agglutination test, and ELISA, are used for brucellosis investigations in many species, and were highly sensitive in detecting infection (Lynch et al., 2011). Therefore, the negative results obtained from dolphins indicate that they have not been exposed to the *Brucella* spp..

This study provides the first documentation of isolates from long-beaked common dolphins of Republic of Korea and it identifies several bacterial species. Although the significance of many of these postmortem isolates is difficult to determine, it is important that we document microorganisms found in long-beaked common dolphins to create a database of potential pathogenic and incidental species. Further research on baseline prevalence, clinical significance, and likely source will help provide data to guide the management of coastal water habitat health to prevent transmission of zoonotic disease.

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Chapter 4

Antimicrobial resistance in bacteria isolated from marine mammals of Republic of Korea

Abstract

The prevalence of antimicrobial resistant bacteria in the marine environment is a growing concern. This study sought to identify the occurrence of antibiotic resistance in bacteria isolated from marinemammals in Republic of Korea. Twenty-four isolates were tested for resistance to antibiotics. Fifty-four percent were resistant to at least one antibiotic, while 17% were resistant to multiple antibiotics. Marine mammals may be important reservoirs of antibiotic-resistant bacteria in the marine environment.

Keywords: antibiotic resistance, bacteria, marine mammals, Republic of Korea

Introduction

Contamination of coastal waters typically carries microbiological pollutants including bacteria, viruses, and protozoa, capable of causing disease in humans and other animals (Oates et al., 2012). Human and animal pathogenic and potentially pathogenic bacteria are constantly released with wastewater into the water environment. Many of these organisms harbor antibiotic-resistance genes, eventually inserted into genetic mobile platforms such as plasmids, transposons, and integrons, able to spread among water and soil bacterial communities (Alonso et al., 2001). Water constitutes not only a way of dissemination of antibiotic-resistant organisms among human and animal populations, as drinking water is produced from surface water, but also the route by which resistance genes are introduced in natural bacterial ecosystems. In such systems, nonpathogenic bacteria could serve as a reservoir of resistance genes and platforms.

The widespread use of antibiotics in human disease treatment and agriculture has resulted in a significant increase in the spread and persistence of antibiotic resistance in the environment (Smith et al., 2002). Antimicrobial resistance can be the result of use or overuse of antimicrobials in clinical and veterinary settings and is a concern to humans and animals as it limits the treatment of infectious diseases and other pathologies.

Despite efforts to reduce the dissemination of antimicrobials into the environment, antimicrobial resistance continues to be of significant concern (Allen et al., 2010). The development of antimicrobial resistance occurs normally in the aquatic environment as bacteria are constantly exposed to antimicrobials selecting for resistance (Zhang et al., 2009). Rapid changes in antimicrobial resistance in aquatic systems are facilitated by the ability of bacteria to transmit resistance genes in a horizontal fashion (Taylor et al., 2011). In addition to antimicrobial exposure, other factors such as metal pollutants and human waste contamination can affect antimicrobial resistance (Baker-Austin et al., 2006; Martinez, 2008).

There are only a few reports of antibiotic resistance in marine animals compared with terrestrial animals, but it has significance with regard to marine mammal stranding and rehabilitation activities, and dissemination of resistant bacteria in the environment. All of these studies have shown that antimicrobial resistant bacteria were consistently recovered from a variety of animals, and that usually more than half of the isolates were resistant to at least one antibiotic.

Currently, not much is known about pathogens that colonize marine mammals in the wild. However, it is important to establish baseline data on bacterial species and traits such as antibiotic resistance that may be of relevance to humans (Ward and Lafferty, 2004). These characterizations are important from a ‘one-health’ perspective recognizing that the health of people, animals,

and our environment are inextricably linked (Kahn et al., 2008; Wallace et al., 2013).

This study was designed to examine patterns associated with antimicrobial resistance in marine animals by surveying bacterial isolates recovered from marine mammals (long-beaked common dolphins and a Steller sea lion) in Republic of Korea.

Materials and Methods

Forty-five marine mammals were collected with the assistance of the Cetacean Research Institute. Stranded and bycaught marine mammals were necropsied and examined to isolate bacteria. From tissue samples, bacteria were isolated, and the Vitek 2 identification system (bioMerieux), 16S rRNA gene analysis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis were performed to identify the isolates (Table 4-1).

Antimicrobial susceptibility was tested by determining MIC using the Sensititre susceptibility system (Trek Diagnostic Systems, West Sussex, United Kingdom) according to the manufacturer's instructions. These tests were conducted in compliance with guidelines from the Clinical and Laboratory Standards Institute (CLSI) with specific selection of antibiotics shown in Table 4-2. Briefly, specific suites of antibiotics were tested depending on species identification.

Table 4-1. Bacterial species identified from marine mammals.

Bacteria	Tissues
Wautersiella falsenii	Ovary
Stenotrophomonas maltophilia	Liver, kidney, vagina
Pseudomonas protegens	Lymph node, lung, stomach
Empedobacter brevis	Uterus, ovary
Acinetobacter johnsonii	Brain, liver, intestine
Acinetobacter haemolyticus	Intestine
Acinetobacter lwoffii	Brain
Myroides sp.	Heart, lung
Aerococcus viridans	Spleen
Lactococcus garvieae	Uterus, kidney
Carnobacterium divergens	Heart, uterus, testis, spleen, liver
Kurthia zopfii	Intestine
Staphylococcus equorum	Kidney
Staphylococcus epidermidis	Lymph node
Staphylococcus cohnii	Lung
Enterococcus pallen	Intestine
Enterococcus faecalis	Intestine
Lactobacillus sakei	Adrenal gland, spleen, thyroid
Lysinibacillus sphaericus	Spleen
Macrococcus caseolyticus	Liver
Streptococcus halichoeri	Vagina
Streptococcus canis	Stomach
Proteus mirabilis	Lung
Mycobacterium porcinum	Lymph node

Table 4-2. Agents used in antimicrobial susceptibility testing of bacterial isolates from marine mammals.

Antibiotic	*Staphylococcus* spp.	*Streptococcus* spp.	*Enterococcus* spp.	Other isolates
Amoxicillin/clavulanic acid				√
Ampicillin		√	√	√
Cefoxitin	√			√
Ceftiofur		√		√
Cephalothin				√
Chloramphenicol	√		√	√
Chlortetracycline		√		
Ciprofloxacin	√		√	√
Clindamycin	√	√		
Colistin				√
Danofloxacin		√		
Daptomycin			√	
Enrofloxacin		√		
Erythromycin	√		√	
Florfenicol		√	√	√
Fusidate	√			
Gentamicin	√	√	√	√
Kanamycin	√		√	

Antibiotic	*Staphylococcus* spp.	*Streptococcus* spp.	*Enterococcus* spp.	Other isolates
Linezolid	√		√	
Mupirocin	√			
Nalidixic acid				√
Neomycin		√		√
Oxytetracycline		√		
Penicillin	√	√		
Quinuoristin/dalfopristin	√		√	
Rifampin	√			
Streptomycin	√		√	√
Spectinomycin		√		
Sulphadimethoxime		√		
Tetracycline	√			√
Tiamulin	√	√		
Tigecycline			√	
Tilmicosin		√		
Trimethoprim/sulphamethoxazole	√			√
Trimethoprim/tulathromycin		√		
Tylosin		√	√	
Vacomycin	√		√	

Results

The dataset consisted of 24 isolates from 45 marine mammals tested for resistance to antibiotics. *Streptococcus* spp. and *Staphylococcus* spp. were sensitive to all tested antibiotics. Fifty-four percent of the total isolates were resistant to at least one antibiotic, while 17% were resistant to multiple antibiotics. While most isolates demonstrated resistance to at least one antibiotic, some also were resistant to multiple antibiotics (Figure 4-1). The total amount of antibiotic resistance observed within individual isolates ranged from 0 to 7 antibiotics. About twenty-one percent of the total isolates were resistant to a single antibiotic, 25% were resistant to two antibiotics and 21% were resistant to three or more antibiotics. Two isolates were resistant to 7 antibiotics.

Detailed information about the bacterial isolates that demonstrated resistance to one or more antibiotics is listed in Table 4-3. The percentage of bacterial isolates demonstrating resistance to each of the 15 antibiotics is shown in Fig.4-2. Three antibiotics were ineffective against 25% of tested isolates: cefoxitin, cephalothin, and tetracycline (Figure 4-2).

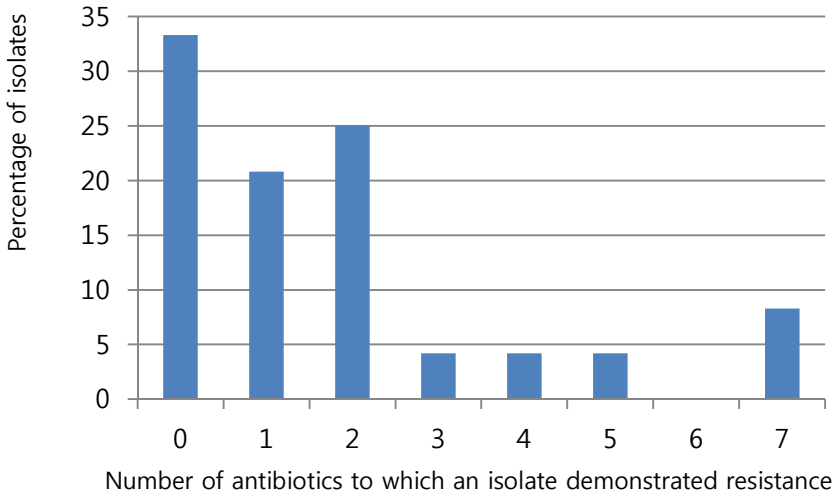

Figure 4-1. Incidence of antibiotic resistance in bacterial isolates from marine mammals.

Table 4-3. Bacterial isolates demonstrating resistance to antibiotics.

Bacterial isolate	Resistance profile
Wautersiella falsenii	AMP, CEP,
Stenotrophomonas maltophilia	AMC, AMP, FOX, CEP, CHL, GEN, TET
Pseudomonas protegens	AMC, AMP, FOX, CEP, CHL
Empedobacter brevis	CEP, GEN
Acinetobacter haemolyticus	AMP, FOX, CEP, CHL
Acinetobacter lwoffii	TET
Myroides sp.	GEN
Aerococcus viridans	SXT
Lactococcus garvieae	TET
Carnobacterium divergens	FOX, NAL, TET
Enterococcus pallen	KAN
Enterococcus faecalis	KAN, QDA
Lactobacillus sakei	FOX, CIP
Macrococcus caseolyticus	FOX, TET
Proteus mirabilis	CHL, TET
Mycobacterium porcinum	AMP, CEP, CHL, COL, FFN, STR, SXT

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEP, cephalothin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; FFN, folrfenicol; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; QDA, quinuoristin/dalfopristin; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulphamethoxazole; TET, tetracycline.

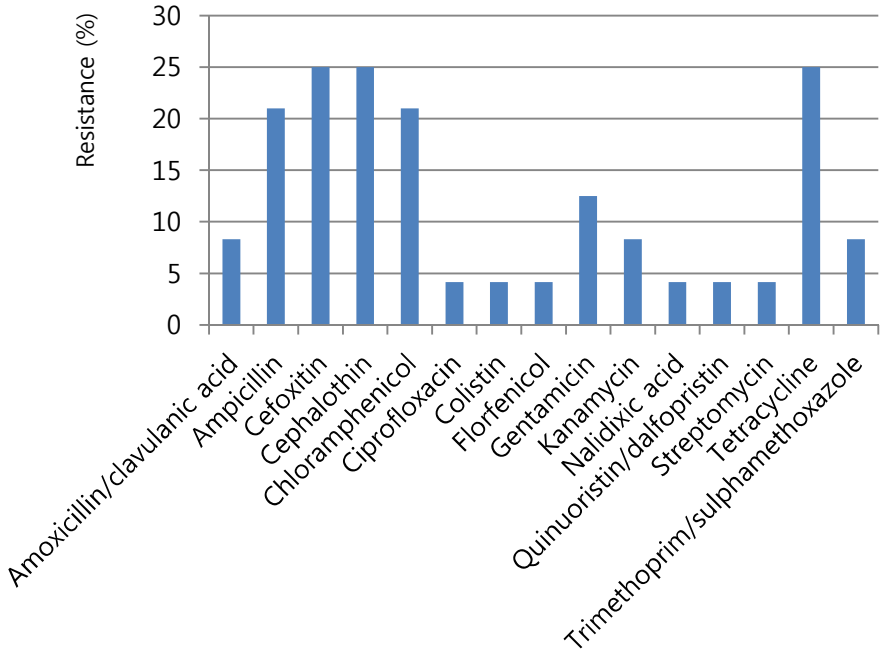

Figure 4-2. Effectiveness of each antibiotic tested against the bacterial isolates.

Discussion

Contamination of coastal waters typically carries microbiological pollutants including bacteria, viruses, and protozoa, capable of causing disease in humans and other animals. Water can transport pathogens and antibiotic-resistant bacteria to areas where the microbial agents are not indigenous (Oates et al., 2012). Antimicrobial use in treatment of humans and food animal husbandry (terrestrial and aquatic) results in the release of wastes that carry both antibiotics and antimicrobial resistant bacteria into the terrestrial and coastal marine environment (Silbergeld et al., 2008).

This study demonstrates widespread antibiotic resistance of bacteria isolated from marine mammals living along the coast of the Korean Peninsula. The antibiotic resistance observed in this study is not the result of therapeutic antibiotic use (i.e. contact with human or veterinary drugs), as all specimens were collected from wild animals. It is known that resistance can be spread by horizontal gene transfer (Neu, 1992). It has also been demonstrated that environmental concentrations of antibiotics can inhibit growth of bacteria, exerting a selective pressure that could result in resistance (Tello et al., 2012). The observation of resistance reported here is consistent with other studies of marine mammals in coastal oceans. Rose et al. (2009) reported widespread antibiotic resistance in marine vertebrates off the northeastern coast of the

United States. Stewart et al. (2014) isolated antibiotic-resistant bacteria from fecal and blow-hole swabs of wild bottlenose dolphins (*Tursiops truncatus*). While this study was successful in identifying and describing the presence of antimicrobial resistant bacteria in marine animals, the number of isolates and samples tested were not usually large enough to allow statistical evaluation of the data. We also do not have direct evidence that the origin of antibiotic resistance in our samples was the coastal environment itself. Without further studies we can only speculate that animals are acquiring antimicrobial resistance from either point sources, such as aquaculture or human wastewater streams, or natural reservoirs of antimicrobial resistance.

Antimicrobial resistant strains isolated from wild marine species are a topic of great concern, since these animals have no history of therapeutic antibiotic exposure (Prichula et al., 2016). Antibiotics represent one of the most prominent aquatic pollutants. The presence of antibiotics in water can cause serious environmental issues, such as the emergence of resistance due to selective pressure. In addition, bacteria from different sources that were likely selected by intensive antibiotic usage are collected and mixed with a number of species, and this mixture with invasive species is likely to cause genetic exchange among micro-organisms (Lupo et al., 2012). Thus, wildlife has the potential to serve as an environmental reservoir by acquiring and dispersing bacterial resistance.

In summary, we observed antibiotic resistance in bacterial isolates from marine mammals in Republic of Korea, and single and multiple antibiotic resistances in this study was consistent with other studies of bacterial isolates of animal origin (Johnson et al., 1998; Bogomolni et al., 2008; Rose et al., 2009; Stewart et al., 2014). The source of antibiotic resistance in bacterial isolates from these marine mammals is not clear. However, the occurrence of single and multiple antibiotic-resistant bacterial isolates from these marine mammals may reflect a large environmental pool of antibicrobial resistant bacteria in coastal waters of Republic of Korea.

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GENERAL CONCLUSION

1. Bacterial examination of the tissues of a male finless porpoise (*Neophocaena asiaeorientalis*) found dead in February 2010 in Tongyeong, Republic of Korea, was performed, and *Dietzia cinnamea* and *Clostridium tertium* were isolated from lung and intestine, respectively. Although *C. tertium* causes clinical diseases in dolphins, we could not determine whether these organisms resulted in any clinical symptoms of the finless porpoise.

2. Out of the isolates from the adult female Steller sea lion, *Streptococcus phocae* and *Streptococcus halichoeri* were known as pathogenic bacteria of marine mammals. However, we could not prove if their infection contributed to the death of the Steller sea lion.

3. Twenty-one species of bacteria were cultured from 44 long-beaked common dolphins, and some species are known as bacterial pathogen to marine mammals and humans. Histopathological examination could not reveal pathogenicity of the isolates on the death of dolphins.

4. The prevalence of antimicrobial resistant bacteria in the marine environment is a growing concern. Among twenty-four isolates tested for

resistance to antibiotics, 54 percent were resistant to at least one antibiotic, while 17 percent were resistant to multiple antibiotics. Marine mammals may be important reservoirs of antibiotic-resistant bacteria in the marine environment.

국문 논문 초록

국내 해양포유동물 폐사의 병인론과 병리학

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우리나라에는 물범과의 점박이 물범과 물개과의 북방물개, 이빨고래아목에 속하는 상괭이와 참돌고래가 분포하고 있다. 물개는 주로 경북연안에서 한두마리씩 발견되는 경우가 대부분이며, 점박이물범은 백령도 주변과 서해연안지역, 남해동부와 동해연안지역에서 발견되고, 밍크고래와 낫돌고래, 참돌고래, 상괭이와 남방큰돌고래가 우리나라 해역에 서식하고 있다. 최근 세계적으로 해양포유동물의 질병에 대한 보고가 증가하고 있다. 우리나라에도 여러 해양포유동물이 분포하고 있으나, 현재까지 해양포유동물 질병에 대한 연구가 이루어지고 있지 않아 본 연구를 시작하게 되었다. 바다에 서식하는 살아있는 해양포유동물에서의 시료채취는 거의 불가능하기 때문에 해안연안에 좌초되거나, 혼획된 해양포유동물을 중심으로 세균성 질병

에 국한하여 연구를 진행하였다. 또한 국내 분리주의 항생제 내성 분포에 대해 분석하였다. 첫번째로 2010년 2월 경남 통영에 표류되어 발견된 상괭이 한마리에 대하여 부검을 실시하고 세균분포와 폐사원인과의 상관성을 밝히기 위해 연구하였다. 그 결과, *Dietzia cinnamea* 와 *Clostridium tertium* 이 각각 폐와 장에서 분리되었다. *C. tertium*은 독소를 생산하지 않는 클로스트리디움으로, 장점막에 콜로니를 형성하여 조직손상을 야기하며 줄무늬 돌고래에서 분리된 보고가 있다. 하지만 이번 연구에서 상괭이에서의 병원성 여부는 밝혀낼수 없었다. 두번째로 2012년 1월 비양도에서 죽은채로 발견된 큰바다사자에 대해 조사하였다. 총 7종의 세균이 분리되었고, 그중 *Streptococcus halichoeri*와 *Streptococcus phocae*는 해양포유동물에 감염성 있는 세균으로 알려져 있다. 하지만 이번 예에서도 큰바다사자의 폐사원인과의 상관성을 밝히기는 어려웠다. 세번째로 2012년 2월부터 2013년 8월까지 강원 삼척과 경북 울진, 영덕, 포항인근 해안에서 혼획된 긴부리 참돌고래 암컷 21마리와 수컷 23마리에 대해 연구를 진행하였다. 긴부리 참돌고래에서 총 21종의 세균이 분리되었고, 이중 해양포유동물뿐만 아니라 사람에도 감염 가능한 인수공통전염성의 세균도 포함되어있었다. 병리학적 검사 결과 정상소견 이외의 소견이 관찰되지 않아 분리주의 병원성 여부는 밝혀낼수 없었다. 국내 해양포유동물에서 분리된 세균의 항생제 내성 분포에 대해

분석한 결과, *Streptococcus* spp. 와 *Staphylococcus* spp. 는 모든 항생제에 감수성을 나타냈지만, 분리주 54%는 하나 이상의 항생제에 내성을 보였으며, 17%가 네개 이상의 항생제에 내성을 나타내어 다제내성균주임을 알수 있었다.

주요어: 긴부리 참돌고래, 상괭이, 세균, 큰바다사자, 한국, 해양포유동물.

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