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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Review

Brucellosis in human and domestic animals in Bangladesh: A review

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According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the World Organization of Animal Health (OIE), brucellosis is considered to be the most widespread zoonosis throughout the world. It is a neglected bacterial zoonotic disease in many countries including Bangladesh. The aim of this study was to review published reports of the brucellosis in humans and domestic animals (cattle, buffalo, sheep, goats, pigs and dogs) in Bangladesh. The prevalence studies are based primarily on the following serological tests: Rose Bengal test (RBT), plate agglutination test (PAT), tube agglutination test (TAT), mercaptoethanol agglutination test (MET), standard tube agglutination test (STAT), slow agglutination test (SAT), milk ring test (MRT), indirect enzyme-linked immunosorbant assay (I-ELISA), competitive ELISA (C-ELISA), complement fixation test (CFT), fluorescent polarization assay (FPA); genus specific and species specific real time PCR. Seroprevalences of brucellosis were found to be affected by the sensitivity and specificity of serological tests employed. Brucellosis prevalence varied based on occupations of people (2.5-18.6%) and species of domestic animals (3.7% in cattle, 4.0% in buffalo, 3.6% in goats and 7.3% in sheep, 4.8% in pigs, 4% in dogs). The prevalence of brucellosis in humans was reported in farmers (2.6-21.6%), milkers (18.6%), butchers (2.5%) and veterinarians (5.3-11.1%) who have direct contact with domestic animals and their products or who consume raw milk. According to published reports, brucellosis does affect people and domestic animals of Bangladesh and there is only one published reports available on the characterization of the *Brucella* isolates of animals in Bangladesh at the species level. There is an immediate need for a concerted effort to control and eradicate brucellosis from domesticated animals in Bangladesh.

Key words: Bangladesh, brucellosis, domestic animals, prevalence.

INTRODUCTION

Early indications of brucellosis date back to the Crimean War (1853-1856) in which *Brucella* spp. was shown as the causative agent of human disease. It was first described in 1859 on the island of Malta by Marston. The first identification of *Brucella* spp. was performed by Dr. Bruce in 1887 and in 1897 Dr. Bang identified *Brucella abortus*. Because of its global expansion, *B. abortus* infection takes different names as Bang's disease, Malta

Figure 1. Aborted fetus from a cow in Bangladesh. The fetus delivered dead at 8 months of pregnancy (c.f. Dey et al., 2013).

fever or undulant fever (OIE, 2014).

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the World Organization of Animal Health (OIE), brucellosis is considered to be the most widespread zoonosis throughout the world (Mustafa and Nicoletti, 1995). This highly contagious zoonotic disease is caused by different species of the genus *Brucella*. These small, Gram negative, non-motile, facultative intracellular, non-spore forming, rod shaped coccobacilli (Baek et al., 2003; Kakoma et al., 2003) are pathogenic for a wide variety of domestic animals including cattle, buffalo, sheep, goat, dog, pig and man (Mathur, 1971). Species of *Brucella* that cause disease in domesticated animals are: *B. abortus* (cattle and buffalo), *Brucella ovis* (sheep), *Brucella melitensis* (sheep and goats), *Brucella suis* (swine) and *Brucella canis* (dog). Brucellosis is primarily a disease of the reproductive tract of domestic animals. The mammary gland is a very important source for transmission of *Brucella* because of its predilection for supramammary lymph nodes and associated shedding in milk. In animals, brucellosis mainly affects reproduction and fertility, with abortion or birth of weak offspring, retention of placenta (Figures 1, 2, 3 and 4) and reduced milk yield. Clinical signs of mastitis are seldom detectable in naturally infected cattle and goats. In man, the clinical picture resembles many other febrile diseases, but sacroiliitis and hepato-splenomegaly are the most prominent symptoms. Severe complications are endocarditis and neurological disorders (Colmenero et al., 1996).

Transmission of *Brucella* to humans results from direct contact with the infected domestic animal, consumption of unpasteurized milk and milk products (Corbel, 2006). Human brucellosis is mainly an occupational disease affecting animal caretakers, farmers, artificial inseminators, abattoir workers, meat inspectors and veterinarians due to frequent exposure to infected domestic animals (Corbel, 2006). Close contact with domestic animals may occur when humans assist animals during parturition or abortion or handling of stillbirth. Farmers and people working in abattoirs frequently have small lesions on their hands that could be the entry point for *Brucella* from infected tissues. Inhalation of *Brucella* has been reported in slaughterhouse workers where the concentration of *Brucella* can be high due to aerosol generation (Sammartino et al., 2005). Dairy farmers who milk animals by hand have a greater chance of becoming infected by the *Brucella* infected animals (Sammartino et al., 2005). Meat inspectors and artificial inseminators who do not take adequate biosafety precautions while performing their jobs are at risk of contracting *Brucella* from the infected animals (Sammartino et al., 2005). Transmission of brucellosis in domestic animal results from ingestion of contaminated feeds and water, inhalation of aerosolized bacteria, sexual intercourse and direct contact with infected placenta and uterine discharges (Corbel, 2006; Radostits et al., 2007). Vertical transmission of *Brucella* is also reported from infected cattle or dam to calf, lambs or kids and other animals (Rahman, 2004; Baek et al., 2005, Rahman and Baek, 2008a). There are two main factors associated with an animal's susceptibility to *Brucella* infection. First, brucellosis primarily affects sexually mature animals (Sammartino et al., 2005). Second, suscep-tibility dramatically increases during pregnancy (Sammartino et al., 2005). Uterine discharge and placenta expelled from infected animals are the main sources of transmission of *Brucella* to humans and animals. Understanding the mode of transmission of *Brucella* is important because it plays a key role in the disease epidemiology. Major risk factors of animal infection are the husbandry practices, local habits and management of the herd/flock. Environmental factors that affect the ability of *Brucella* to survive outside the mammalian hosts are to be considered in the epidemiology of brucellosis. High humidity, low temperature and absence of direct sun light may favor survival of

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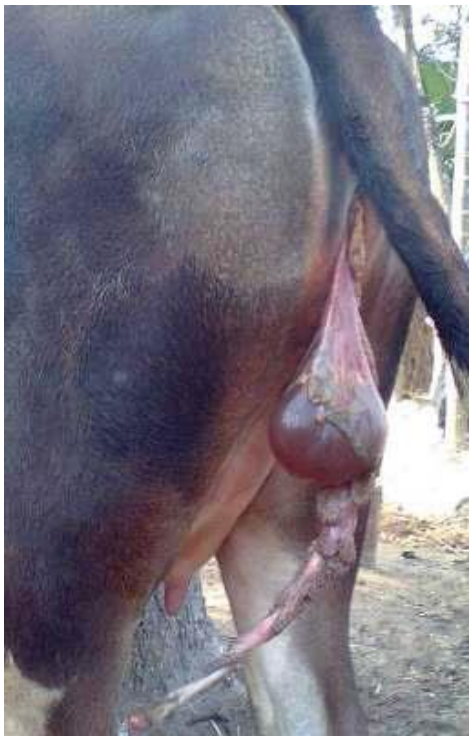

Figure 2. The placenta of the cow failed to discharge after 24 hours of abortion in Bangladesh (c.f. Dey et al., 2013).

Figure 3. Abortion in black Bengal goat from Bangladesh.

Brucella for several months in water, aborted fetuses, placental membranes, liquid manure, hay, buildings, equipment and clothes (Sammartino et al., 2005).

Figure 4. Abortion in sheep from Bangladesh.

Human brucellosis poses major economic and public health challenges in affected countries especially in the Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico, and Central and South America (Pappas et al., 2006). Human brucellosis remains the most common zoonotic disease worldwide, with more than 500,000 new cases reported annually (Pappas et al., 2006). Globally this disease is woefully under-reported because of its vague clinical flu like symptoms, difficulty in laboratory diagnosis and lack of familiarity by medical professionals (Corbel, 2006). Therefore, the true incidence of human brucellosis is unknown for most developing countries of the world including Bangladesh. Data documenting human brucellosis are very meager in Bangladesh. Published reports indicate that it is an occupational disease among farmers, milkers, butcher and veterinary practitioners in Bangladesh (Nahar and Ahmed, 2009; Muhammad et al., 2010; Rahman 2011a).

Diagnosis of brucellosis in humans and domestic animals is mainly based on detection of *Brucella* lipopolysaccharide (LPS) specific antibodies in milk and serum samples using serological tests. Serological tests are commonly used for *Brucella* diagnosis in cattle and small ruminants at herd level. The sensitivity of RBT fulfills the requirements for surveillance at flock level (European Commission Regulation, 2002). The SAT is an easy to use screening method with a good sensitivity but lower specificity. CFT is an OIE mandatory test for international trade (Nielsen, 2002). None of the above

mentioned tests can distinguish between antibodies produced after vaccination and those due to field infection (Nielsen et al., 1989). Different enzyme-linked immune-sorbent assays (ELISA) have been developed to overcome these problems and are capable of detecting *Brucella* carriers being seronegative by RBT, SAT and CFT (Van Aert et al., 1984). Nowadays, real time (RT) PCR methods are used to amend serological diagnostics. DNA of *Brucellae* can readily be detected from serum of infected animals even if blood culture fails. Additionally, species differentiation out of serum using IS711 species specific RT PCR is possible (Rahman et al., 2013a). The genomes of several *Brucella* species have been sequenced and different molecular methods including multiple locus variable number repeat analysis (MLVA) has been developed for species identification and subspecies genotyping (Le Fleche et al., 2006). Genotyping may be used to study the diversity of genotypes and to trace source of infection (Kattar et al., 2008).

In this review, we summarized the published literatures on human and domestic animal brucellosis in Bangladesh and we recommended strategies to control brucellosis in Bangladesh.

BRUCELLOSIS IN HUMAN

Approximately 2.4 billion people are at risk of getting brucellosis every year in the world. Bangladesh is situated in the northern part of south Asia between 20°38′ and 26°38′ north latitude and between 88°01′ and 92°41′ east longitude. Bangladesh has one of the highest popu-lation densities in the world (1015 per sq km). It has 147,570 km^2 area of land with seven divisions.

Rahman (1983) conducted the first sero-prevalence study of brucellosis in humans in Bangladesh. This study recorded 12.8% prevalence of brucellosis in dairy and agricultural workers and 21.6% prevalence among goat farmers.

Nahar and Ahmed (2009) carried out a seroprevalence study using RBT and STAT on 50 human sera. The study recorded *Brucella* positive specimen in animal owners (1 of 7), animal attendants (1 of 13) and veterinary students (1 of 26). Muhammad et al. (2010) analyzed 210 human sera of people at risk in the Mymensingh district using a variety of *Brucella* serological tests. Seroprevalences among occupational groups were 11.1% in veterinary personnel, 6.5% in dairy workers and 4.7% in animal farmers.

Rahman et al. (2012a) conducted a study to determine the seroprevalence of brucellosis in a high-risk exposure group of individuals (n = 500). The prevalence of brucellosis was 2.6% in farmers, 18.6% in milkers, 2.5% in butchers and 5.3% in veterinary practitioners. The prevalence was higher in males (5.6%) than females (0.8%). The highest prevalence was recorded in Dhaka district (24%) followed by Mymensingh district (2.9%). Higher prevalence was recorded in farmers handling goats (8.5%) as compared to farmers handling cattle and goats (4.7%) or cattle only (3.5%). The prevalence was higher in individuals with the history of drinking raw milk (11.4%) than individuals not drinking raw milk (3.9%). The highest prevalence was recorded in individuals (16.2%) having contact with animals for more than 26 years. The prevalence was higher in 41-80 years age group (6.2%) followed by 21-40 years group (3%) and 14-20 years age group (2.3%), respectively. The study emphasized that contact especially with goats, is a significant risk factor for infection with *Brucella* of individuals in high-risk group.

The results of all seroprevalence studies indicated that brucellosis is an occupational health hazards in Bangladesh among milkers, farmers and veterinarians. The type of animals owned or handled, and duration of contact with domestic animals and consumption of raw milk are the risk factors associated with brucellosis in humans in Bangladesh (Rahman et al., 2012a).

BRUCELLOSIS IN CATTLE

Cattle constitute the major domestic animal in Bangladesh. Most of the households in the villages of Bangladesh rear cattle and Bangladesh has 23.4 million cattle. Cattle reared in Bangladesh are mainly indigenous zebu, some exotic breeds and their crosses predomi-nantly Holstein-Friesian, Jersey, Sahiwal and Sindhi. Dairying is part of the mixed farming systems and a predominant source of income, nutrition and jobs and a strong tool to develop a village micro economy of Bangladesh in order to improve rural livelihoods and to alleviate rural poverty. One of the infectious diseases, which are a major constraint for dairy animal productivity, is brucellosis. Brucellosis in dairy cattle is caused by *B. abortus* (Rahman, 2011a, b)

Brucellosis in cattle in Bangladesh was first reported by Mia and Islam (1967). Prevalence of brucellosis in cattle was demonstrated as 18.4% (Rahman and Mia, 1970). Prevalence was also reported from milk samples in dairy farms as 11.4, 11.7 and 4.2% in Savar, Tangail and BAU dairy farms, respectively (Rahman et al., 1978). Milk samples of cattle provided 5.5 and 11.4% prevalence rates of brucellosis in BAU dairy farm and central cattle breeding and dairy farm (CCBDF) Savar, respectively (Rahman and Rahman, 1981).

Prevalence of brucellosis in cows on dairy farms of Pabna, Faridpur and Bogra districts were 11.5, 2.9 and 2.0%, respectively (Rahman and Rahman, 1982). The annual economic loss in Bangladesh due to bovine brucellosis in indigenous cows was 720,000 EUR (total) and 12000 EUR per 1000 cross-bred cows and a total of 276000000 EUR in cross-bred cows in Bangladesh (Islam et al., 1983).

Islam et al. (1992) recorded 15% prevalence of brucellosis in exotic breed of cows and 9% in local cattle breed after screening 760 sera of cows from Avoynagar, Puthia, Hazirhat, Comilla, Manikgonj and Moshurikhola of

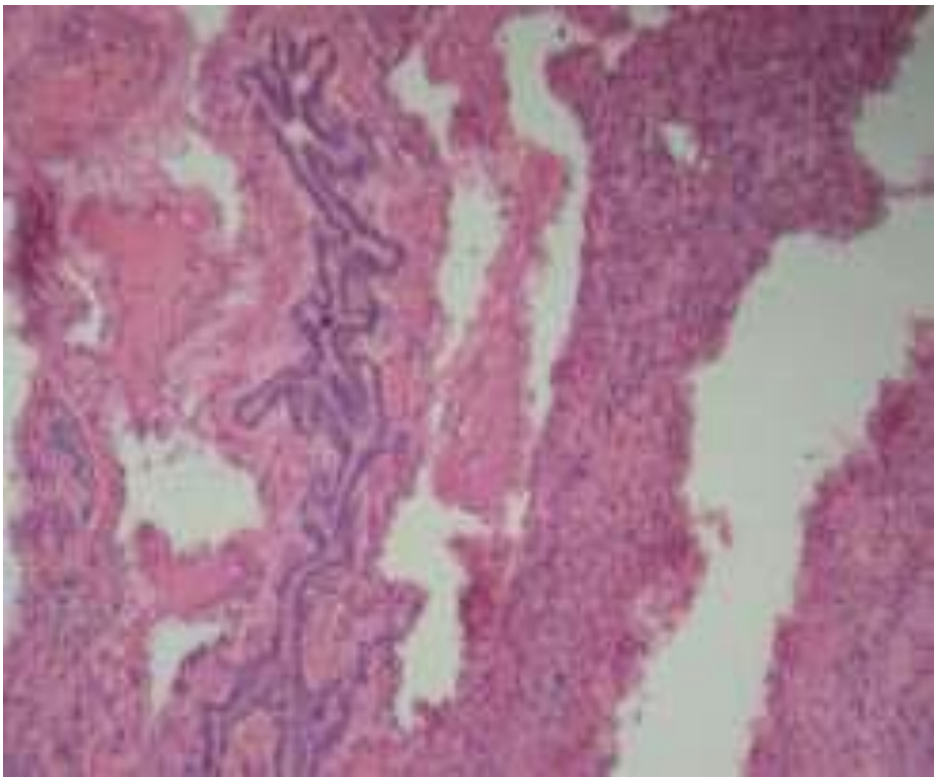

Figure 5. A piece of placenta obtained from the cow with a history of abortion and seropositivity to RBPT and I-ELISA. There was diffuse fibrosis around the placental epithelium and in placental tissues (H & E, 10x) (c.f. Dey et al., 2013).

Bangladesh by rapid screening test and tube agglutination test (TAT). Ahmed et al. (1992) reported 5% prevalence of brucellosis in dairy farms and 2.8% prevalence in rural cows by plate agglutination test (PAT) and TAT. This study recorded 3.2% prevalence of brucellosis in pregnant cow and 3.1% in non-pregnant cows. Prevalence of brucellosis was higher in cows above 3 years age (4.8%) than cows less than 3 years (0.7%). The prevalence of brucellosis was 9.1% in cows with a history of previous abortion.

Rahman et al. (2006) reported the prevalence of brucellosis and its association with reproductive problems in cows in Bangladesh. The prevalence of the disease among the 260 rural cows was 3·08% by the RBT and the PAT, and 1·92% by the TAT. The difference between the two groups was not statistically significant. In cows with a history of retained placenta, the prevalence of brucellosis was 13·04% by the RBT and PAT, and 8·70% by the TAT. The prevalence of brucellosis in repeat breeding cases was 1·45% by all three serological tests. There was a statistically significant difference in the prevalence of the disease between cows with a history of retained placenta and repeat breeding cases ($P<0·05$). The prevalence of brucellosis in cows that had mastitis was 4·76% by the RBT and PAT, but was not demonstrated by the TAT.

During the period of 2004-2012, a total of 1487 serum or milk samples were obtained from cattle in six districts of Bangladesh and overall prevalence of brucellosis 4.2% was found in Mymensingh, 8% in Dinajpur, 1.1% in Bagherhat, 5% in Chittagong and 0% both in Bogra and Gaibandha districts (Amin et al., 2004; Nahar and Ahmed, 2009; Ahasan et al., 2010; Rahman et al., 2012b; Sikder et al., 2012). Dey et al. (2013) recorded serological and pathological investigations of brucellosis in dairy cows in Bangladesh. Out of 190 randomly sera sample tested, prevalence was 2.63% by RBPT and 1.05% by I-ELISA. Histopathological study of placenta from an aborted cow and spleen and lymphnode and liver from an aborted fetus were performed. During histopathological study there was depletion of lymphocytes in spleen and lymphnodes which was characterized by reducing densities of lymphocytes. The smooth muscular trabeculi in spleen and fibromuscular trabeculi in lymphnode were distended. In placenta, there was diffuse fibrosis around the placental epithelium. The liver of aborted fetus showed multifocal necroses in hepatic parenchyma and necrosed tissue was replaced by fibrous connective tissue and reactive cells (Figures 5, 6, 7 and 8).

Rahman et al. (2013a) recorded 5.29% prevalence of brucellosis in 700 cattle sera by RBT. RBT positive samples were retested by CFT, SAT, ELISA and real

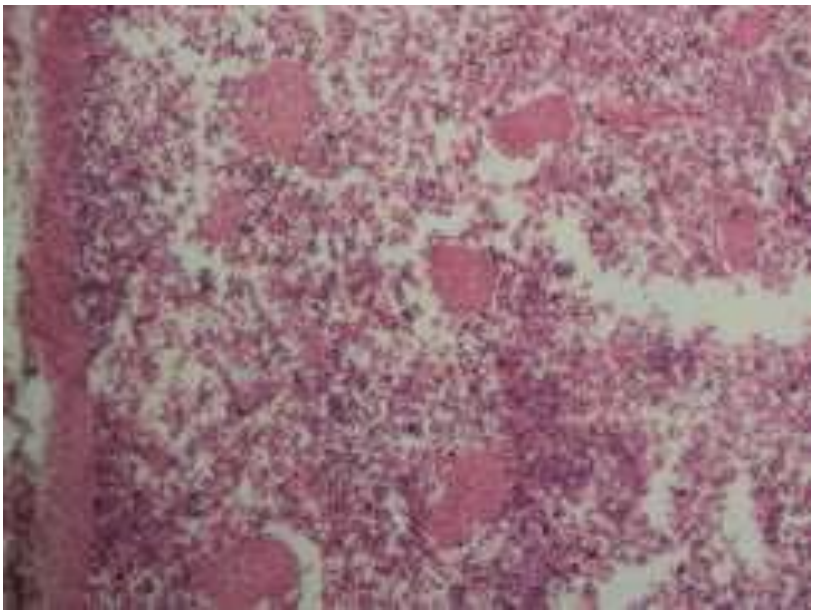

Figure 6. Spleen obtained from a dead calf delivered from *Brucella* seropositive cow and stained with H & E. There were depletion of lymphocytes in spleen and distended trabeculi (10x) (c.f. Dey et al., 2013).

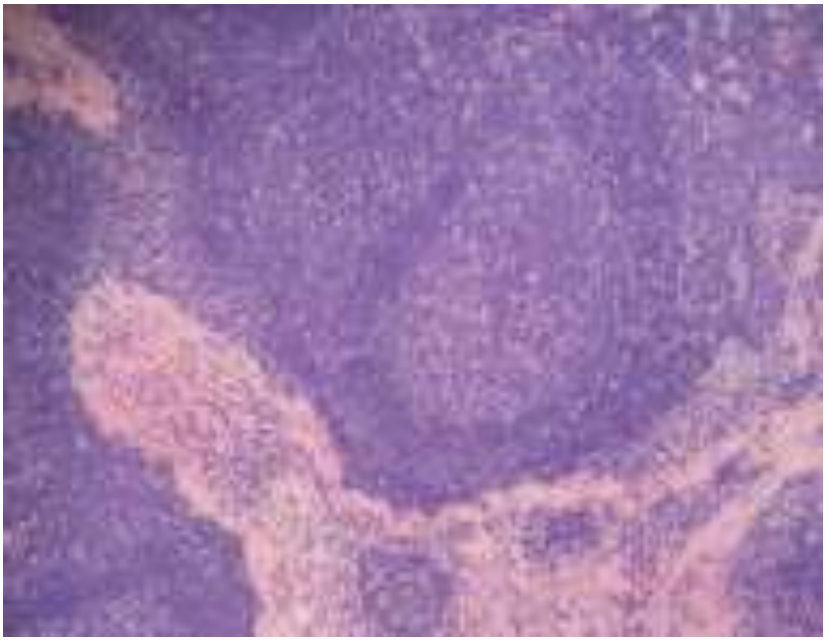

Figure 7. Lymph node obtained from a dead calf delivered from *Brucella* seropositive cow and stained with H&E. There were depletion of lymphocytes in lymphnode and distended trabeculi (10x) (c.f. Dey et al., 2013).

time PCR. *Brucella* DNA was found in 4.43 % of the cattle by genus specific real time PCR of which *B. abortus* DNA was found in 1.9% of cattle by species specific real time PCR in Kurigram and Mymensingh districts of Bangladesh (Figure 9) (for amplification of real time PCR). Rahman et al. (2013b) reported the prevalence and diagnostic test comparison of brucellosis in cattle in Pabna and Mymensingh districts of Bangladesh. The seroprevalence of brucellosis in cattle was estimated to be 4.23, 3.07 and 2.31% by RBT, SAT and I-ELISA,

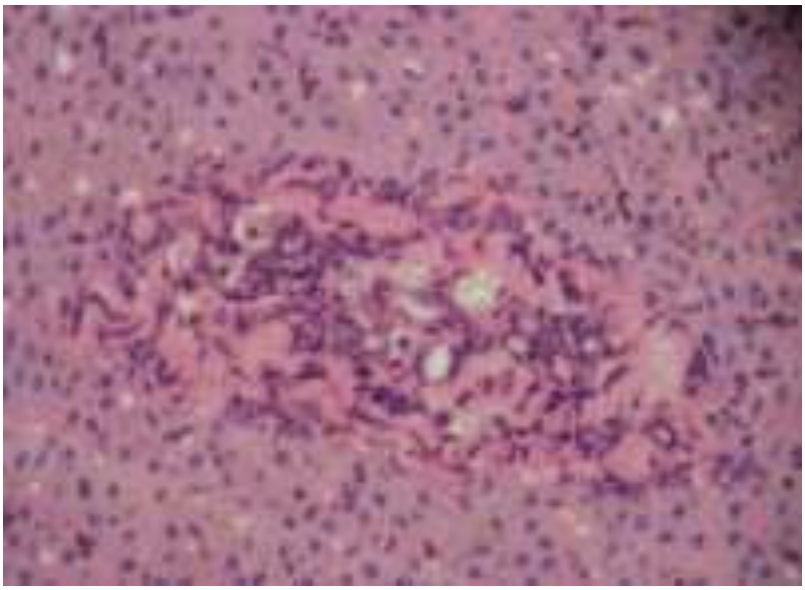

Figure 8. Section of a liver collected from a dead calf suspected to BE infectED with *Brucella*. There were multifocal necrosis in hepatic parenchyma and necrosed tissue was replaced by fibrous connective tissue (H & E, 40x) (c.f. Dey et al., 2013).

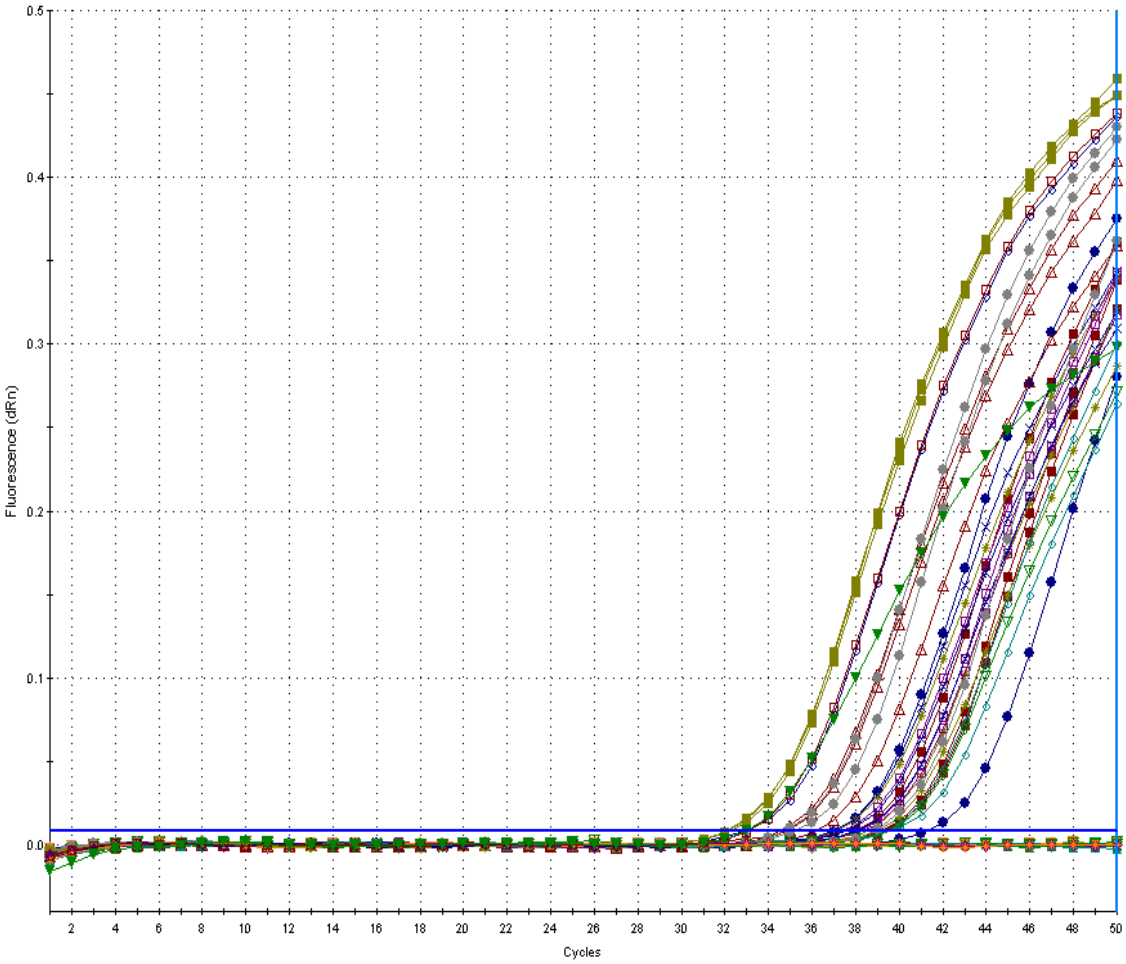

Figure 9. Amplification plots in real time PCR with the cattle and buffalo sera of Bangladesh.

Table 1. Prevalence of brucellosis in cattle in seven districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Mymensingh	250	5	2.00	Amin et al. (2004)
Mymensingh	200	9	4.50	Nahar and Ahmed (2009)
Mymensingh	132	14	10.60	Ahasan et al. (2010)
Mymensingh	100	7	7.00	Rahman et al., (2013a)
Mymensingh	190	5	2.63	Dey et al. (2013)
Dinajpur	50	4	8.00	Ahasan et al. (2010)
Mymensingh	135	2	1.50	Rahman et al. (2012b)
Bogra	60	0	0.00	Rahman et al. (2012b)
Gaibhandha	70	0	0.00	Rahman et al. (2012b)
Bagherhat	90	1	1.10	Rahman et al. (2012b)
Chittagong	500	25	5.00	Sikder et al. (2012)
Kurigram	600	30	5.00	Rahman et al., (2013a)

respectively. The comparison of the serological tests result revealed the highest prevalence in RBT than SAT and I-ELISA. The prevalence of *Brucella* was 2.5% in Pabna and 2.14% in Mymensingh. It was observed that, a higher prevalence of *Brucella* was found in female (2.67%) than in male (1.82%), natural breeding (2.67%) than artificial breeding (1.81%), in aged animals (3.33%) than young (1.25%). But these differences were not statistically significant. There exists significant difference between prevalence of brucellosis in cattle with history of abortion than without history of abortion. See Table 1 for prevalence of brucellosis in cattle in different districts of Bangladesh.

BRUCELLOSIS IN BUFFALO

Asia is the native home of the water buffalo, with 95% of the world population, with about half of the total in India and Bangladesh. It is valuable for its meat and milk, as well as the labour it performs. It is often referred to as “the living tractor of the East”, as it is relied upon for plowing and transportation in many parts of Asia including Bangladesh (Rahman, 2012a).

Buffaloes are known to be affected with *B. abortus* and less frequently with *B. melitensis* (Munir et al., 2008; Ahmed et al., 2010). Similar to cattle, *Brucella* infections are known to result in late gestation (6-9 months) abortions (Sanjrani et al., 2013), infertility (Sukumar et al., 2012) and latent infection of mammary gland lymph nodes with shedding of organisms in the milk (Ahmed et al., 2010), yet abortions are less common in buffaloes (The Center for Food Security and Public Health Iowa State University, 2009) with the disease being endemic in most buffalo raising countries. Shedding of *Brucella* in milk creates a potential threat to human health particularly for consumers using unpasteurized milk and milk products (Ahmed et al., 2010). A slightly lower incidence of brucellosis has been recorded in buffaloes as compared to cattle in studies that simultaneously evaluated the serologic presence of brucellosis in these two species (Hussain et al., 2008), however, in other studies, a higher incidence of the disease was recorded in buffaloes as compared to cattle (Nasir et al., 2004). Thus, it can be presumed that buffaloes are differentially affected with *B. abortus*.

The first report on the occurrence of brucellosis in buffaloes appears to have originated in India in 1918 at the Indian Veterinary Research Institute, Mukteshwar (Anonymous, 1918). The first seroprevalence study of brucellosis in buffalo in Bangladesh was conducted by Rahman et al. (1997) in selected areas of Bangladesh. The overall seroprevalence in buffalo was 6.9% by PAT and 2.4% by TAT. The prevalence of brucellosis was 7.1 and 1.2% in buffalo with history of retained placenta and repeat breeding, respectively.

Rahman et al. (2012b) screened 135 sera of buffaloes from five districts of Bangladesh and found prevalence of brucellosis in Bagherhat, Mymensingh and Sirajgonj districts as 2.9, 8.3 and 5.3%, respectively. No prevalence of brucellosis was recorded in buffalo both in Bogra and Gaibandha districts. Age and sex as potential risk factors for brucellosis in buffalo was analyzed by Rahman et al. (2011a). Recently Rahman et al. (2013a) recorded prevalence of brucellosis in 99 buffaloes sera of Bagerhat and Mymensingh using RBT, SAT, CFT, I-ELISA, genus specific and species specific real time PCR (see Figure 9 for amplification of real time PCR). The presence of *Brucella* DNA was found in 7.1 % of the buffaloes investigated and *B. abortus* DNA was found in 6.1% of the buffaloes. Prevalence of brucellosis in buffaloes in different districts of Bangladesh is shown in Table 2.

BRUCELLOSIS IN GOAT

Economically and culturally, the goat has played an important role in traditional Bengali society. Among the Asiatic countries, Bangladesh, a tropical agro-based developing country, possess the third largest repository

Table 2. Prevalence of brucellosis in buffalo in five districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Bagherhat	70	2	2.85	Rahman et al. (2012b)
Bagherhat	80	5	6.50	Rahman et al. (2013b)
Bogra	20	0	0.00	Rahman et al. (2012b)
Gaibandha	14	0	0.00	Rahman et al. (2012b)
Mymensingh	12	1	8.33	Rahman et al. (2012b)
Mymensingh	19	2	10.52	Rahman et al. (2013a)
Sirajgonj	19	1	5.26	Rahman et al. (2012b)

Table 3. Prevalence of brucellosis in goats in eight districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Mymensingh and Dhaka	300	5	1.67	Uddin et al. (2007b)
Dhaka and Lalmonirhat	20	0	0.00	Das et al. (2008)
Mymensingh	208	8	3.85	Islam et al. (2010)
Bogra and Mymensingh	120	7	5.83	Rahman et al. (2011b)
Bagherhat	15	1	6.67	Rahman et al. (2012b)
Bogra	30	0	0.00	Rahman et al. (2012b)
Gaibandha	50	2	4.00	Rahman et al. (2012b)
Mymensingh	100	4	4.00	Rahman et al. (2012b)
Sirajgonj	35	1	2.86	Rahman et al. (2012b)
Nilphamari	154	5	3.24	Rahman et al. (2012c)

of goats, with a population of more than 34 million heads, according to the FAO (WHO, 2006). This figure represents more than 57% of total livestock in Bangladesh. More than 90% of the goats of the country are of the Black Bengal breed. Each year goat production provides 127,000 MT meat, which accounts for 25% of total red meat in Bangladesh (Bangladesh Economic Review, 2012). As goats come in very close contact with humans, the risk of transmitting this zoonosis is very high (Rahman, 2012b).

Serological prevalence (14.5%) of brucellosis in goats in Bangladesh was first reported by Rahman (1983). A higher incidence of the disease was observed in goats with reproductive disorders (Rahman et al., 1988). Overall prevalence of brucellosis in goats were 1.7% in Mymensingh and Dhaka districts, 0% in Dhaka and Lalmonirhat districts, 3.9% in Mymensingh district, 5.8% in Bogra and Mymensingh districts, 6.7% in Bagherhat districts, 0% in Bogra district, 4% in Gaibandha district and 2.9% in Sirajgonj district (Uddin et al., 2007a, b; Das et al., 2008; Islam et al., 2010; Rahman et al., 2011b, 2012b). Potential risk factors for brucellosis in goats included age, sex, pregnancy status, management system (concrete floor versus mud floor; flock rearing vs. individual rearing; non grazing versus free grazing; mixed with cattle versus without cattle; and rural versus farm goats) and reproductive disorders (abortion vs. retained placenta). Prevalence odds of brucellosis in goats that are pregnant were 7 times greater than the prevalence odds of brucellosis for goats that are not pregnant. Rahman et al. (2012c) found overall seroprevalence of brucellosis 59% in Black Bengal goats in Nilphamari district of Bangladesh. A significantly ($p<0.01$) higher prevalence of brucellosis was found in Black Bengal goats with the history of previous abortion (33.33%). An insignificant ($p>0.05$) but higher prevalence of brucellosis was found in adult Black Bengal goats (>24 months) than young ones. The prevalence was relatively higher in cross-bred than pure Black Bengal goats, in female than male and in pregnant than non-pregnant Black Bengal goats. Prevalence of brucellosis in goats in different districts of Bangladesh is shown in Table 3.

BRUCELLOSIS IN SHEEP

Among the livestock populations, sheep still occupies the third position and about 80% sheep is reared by rural farmers in Bangladesh. The sheep in Bangladesh are mainly indigenous and utilized for meat purposes but also important for good quality leathers and source of income to rural people. In Bangladesh, sheep and goats are a very valuable asset especially for poor people. Most cases of brucellosis infection in sheep are inapparent and lack

Table 4. Prevalence of brucellosis in sheep in six districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Mymensingh and Dhaka	62	3	4.84	Uddin et al. (2007a)
Bogra and Mymensingh	80	3	3.75	Rahman et al. (2011b)
Bagherhat	27	3	11.11	Rahman et al. (2012b)
Bogra	30	1	3.33	Rahman et al. (2012b)
Gaibandha	35	2	5.71	Rahman et al. (2012b)
Gaibandha	206	7	3.39	Rahman et al. (2012d)
Mymensingh	40	8	20.00	Rahman et al. (2012b)
Sirajgonj	38	2	5.26	Rahman et al. (2012b)

Table 5. Prevalence of brucellosis in pigs in 2 districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Bogra	62	3	4.80	Rahman (2011c)
Bogra	63	4	6.60	Rahman et al. (2012e)
Sirajgonj	41	2	4.80	Rahman (2011c)
Sirajgonj	42	3	7.10	Rahman et al. (2012e)

clinical signs. Serological evidence of brucellosis in sheep in Bangladesh was first reported by Uddin et al. (2007b). *Brucella* antibodies were prevalent in 8.84% sheep.

The overall prevalence of brucellosis in sheep (n = 312) reported by Rahman et al. (2011b, 2012b) from Mymensingh and Dhaka, Bogra and Mymensingh, Bagherhat, Bogra, Gaibandha, Mymensingh and Sirajgonj districts was 4.8, 3.8, 11.1, 3.3, 5.7, 20 and 5.3%, respectively. Prevalence odds of brucellosis in sheep that are greater than 2 year of age were 90 times greater than the prevalence odds of brucellosis for sheep that are less than or equal to 2 years (Rahman et al., 2011a, b). Further investigation by Rahman et al. (2012d) recorded seroprevalence of brucellosis in sheep in the Gaibandha districts of Bangladesh as 3.39% by RBPT and 2.91% by i-ELISA. The prevalence of brucellosis was higher in female sheep (3.41%) than male (3.33%) and in sheep with history of abortion (4.34%) than without history of abortion (3.08%). The higher rate (4.59%) of *Brucella* antibody was recorded in sheep of 1-2 years of age. Prevalence of brucellosis in goats in different districts of Bangladesh is shown in Table 4.

BRUCELLOSIS IN PIGS

Brucellosis in pigs is caused by *B. suis*. The capability of *B. suis* to colonize the bovine udder with subsequent shedding in milk means that it has the potential to be a serious human health risk. Outbreaks in slaughter houses have been caused by inhalation of *B. suis*. Most cases occur in people employed in meat processing industry and animal rearing (Radostits et al., 2007). Though, out of 590 million pigs in the world, about 34% are raised in tropical countries. From to the religious point of view and for the limited number of pork consumers, the pig population is not large as compared to other ruminants and birds in Bangladesh. Furthermore, it is difficult to get the exact number of pigs in Bangladesh. But the pig population is increasing in the tribal areas. The pig rearing continues to be primitive scavenging in nature because they are raised by certain rural people who are educationally, economically and socially most backward. Serological evidence of brucellosis in pig in Bangladesh was first reported by Rahman (2011c).

Further serological status of brucellosis in pigs was diagnosed by Rahman et al. (2012e) in Bangladesh using RBT and SAT. Overall seroprevalence was 6.7 and 4.8% by RBT and SAT, respectively. It was observed that, insignificantly higher prevalence of brucellosis based on SAT was found in female (5.6%) than male (2.9%) in aged animal (8.1%) than young (0.0%) and in pregnant animal (12.5%) than non pregnant animal (2.1%) ($p>0.05$). Prevalence of brucellosis was 42.9% in aborted pigs and 1.6% in non aborted pigs. The association between abortion status and prevalence of brucellosis was statistically highly significant ($p<0.01$). Prevalence of brucellosis in pigs in different districts of Bangladesh is shown in Table 5

BRUCELLOSIS IN DOGS

Dogs fill a variety of roles in human society and are often trained as working dogs. The most important role of dogs is as companion. Dogs have lived with and worked with humans in so many roles that their loyalty has earned

Table 6. Prevalence of brucellosis in dogs in 2 districts of Bangladesh.

District	No. tested	No. positive	Positive (%)	Reference
Mymensingh	30	4	13.33	Talukder et al. (2011)
Dhaka	50	2	4.00	Rahman (2014b)

them the sobriquet man's best friend. Dog population in Bangladesh may be considered as a carrier of *Brucella* infection and might act as a risk for food animal and human health (Rahman, 2014a). Dogs may become infected through ingestion of infected bovine placental tissue. *Brucella* infected dogs may abort and vaginal discharges have a potential for transmitting *Brucella* to susceptible animals. Both *B. abortus* and *B. melitensis* infection have been reported in dogs kept on farms (Baek et al., 2003).

The first report on the sero-prevalence of brucellosis in stray dogs of Bangladesh by using four commercial sero-diagnostic kits was conducted by Talukder et al. (2011). The overall sero-prevalence of canine brucellosis was recorded as 13.33, 6.67, 6.67 and 10.0% with RBPT, SAT, STAT and ELISA, respectively. Significantly ($p<0.01$) higher sero-prevalence rate of canine brucellosis was recorded in stray dogs aged between 7 and 36 months (14.81, 7.40, 7.40 and 11.11%) in comparison with aged group up to 6 months (0, 0, 0 and 0%) with RBPT, SAT, STAT and ELISA, respectively. The sero-prevalence rate of canine brucellosis was found significantly ($p <0.01$) higher in female dogs (15.78, 10.52, 10.52 and 15.78%) in comparison with male (9.09, 0, 0 and 0%) with RBPT, SAT, STAT and ELISA, respectively.

Rahman (2014b) conducted a serological study for a total of 50 pet dog's serum samples collected from Dhaka, Bangladesh. The overall seroprevalence of brucellosis in pet dogs was found to be 4.00%. Statistically significant higher seroprevalence of brucellosis (RBPT and ELISA, 6.06% respectively) was found in dog aged 1.5 to 2.5 years. Higher seroprevalence (15.38%) was found in female pet dogs and no response in male pet dogs. Prevalence of brucellosis in dogs in different districts of Bangladesh is shown in Table 6.

Recommended strategy to control brucellosis in Bangladesh

It is important to remember that brucellosis is an important zoonosis and nearly every case of human brucellosis has an animal origin and, therefore, control is primarily a veterinary responsibility (Nicoletti, 1992). The *Brucellae* are 'survivors' in both extracellular and intracellular environments. Compatible relationships with the hosts including variable incubation periods, asymptomatic carriers and resistance to treatments are the important problems. The animal husbandry factors such as commerce, nomadism, commingling and increasing population sizes assure difficulties in control of diseases.

The serosurveillance studies of brucellosis in humans and animals suggest that brucellosis is endemic in the surveyed areas of Bangladesh. Without control measures, the infected domestic animals will continue to serve as reservoirs for the spread of the disease to uninfected domestic animals and humans.

CONTROL OF BRUCELLOSIS IN HUMANS

Public health education

Efforts should be focused on the public health education regarding the disease and its risk factors. The duration of contact with animals and the type of animal handled appeared to be the most significant risk factors for human brucellosis in Bangladesh (Rahman et al., 2012a). Exposure could be minimized by educating individuals within the high risk group (Rahman et al., 2012a).

Food safety

Brucella spp. are readily killed by pasteurization or heating of raw milk. Pasteurization process is not available in all parts of Bangladesh. Boiling or heating of milk at 80-85°C (176-185 8°F) for several minutes will kill the *Brucella* (Corbel, 2006).

Personal hygiene

Protective clothes such as overalls, rubber gloves and rubber boot should be used during handling of domestic animals. If gloves are not available, washing of hands with soap and water immediately after examination is recommended. Consuming of food and smoking must be forbidden in the abattoirs while handling domestic animals (Sammartino et al., 2005).

Improved diagnostic and treatment facilities

Brucellosis in humans is under-reported globally (Corbel, 2006) and likely under-reported in Bangladesh as well. Due to the scarcity of diagnostic and medical tools, treatment of brucellosis is often not possible. Appropriate test facilities for early and accurate diagnosis of brucellosis and prescription of effective antimicrobial treatment regimen must be included in the human health care system of Bangladesh.

Collaboration between human and veterinary medicine

Control of brucellosis in domestic animals is the key to decreasing human cases since it is transmitted to humans from infected domestic animals and their products (Jiang and Baldwin, 1993). Collaboration between the department of health and department of livestock services are important to control brucellosis in domestic animals and thereby eliminate transmission to humans. Veterinary medicine must implement methods to control/eradicate brucellosis in domestic animals while human medicine must develop complementary methods to prevent transmission and develop effective treatment of human patients. So it is critical that physicians and veterinarians cooperate in these efforts.

CONTROL OF BRUCELLOSIS IN DOMESTIC ANIMALS

Surveillance program

Surveillance is important for determining prevalence and thereby allow for the development of preventive and control measures and eventual eradication of brucellosis in domestic animals. Brucellosis is primarily diagnosed by serological tests and rapid screening tests can be done by either RBT or PAT in the field. Conventional serological tests like rivanol, 2-MET and complement fixation tests (CFT), I-ELISA, C-ELISA and FPA are used as confirmatory tests. An excellent surveillance option is testing bulk tank milk samples among dairy herds by MRT (Sarker et al., 2014). The appropriate places for testing animals are slaughterhouses, livestock markets or any livestock sale station. This surveillance will help trace-back the infected animals to the herd or flock of origin. The polymerase chain reaction (PCR) can be used for identification of *Brucella* species or biovars and would be useful for epidemiological trace-back in a brucellosis control program (Rahman et al., 2013a).

Control of unrestricted animal movements

The initial introduction of disease into a herd or flock is often due to replacement animals introduced from an infected herd or flock of unknown disease status (Crawford et al., 1990). Implementation of quarantine and serosurveillance of the new replacement animals before they enter the farms and checking the imported animals at border check points before entering into the country are required to ensure that these animals are free from brucellosis.

Epidemiological investigations

Animal age, sex, gestation stage, virulence of the pathogen, environmental conditions affect exposure to infection (Nicoletti, 1984). A detailed epidemiological investigation focusing on host, agent and environment factors needs to be performed throughout the country in order to identify the risk factors associated with transmission and maintenance of brucellosis in animals.

Investigation of causes of abortion

Making animal abortion notifiable and investigations into the causes of abortion help identify not only *Brucella* but also allow for the identification of other causative agents. This method of detection relies on compliance by farmers and veterinarians provided enough resources are available to conduct investigations following a report of an abortion (Crawford et al., 1990).

Improved animal management practices

The practice of mixing of cattle, either through grazing or sharing of watering points, is a significant risk factor for brucellosis (Crawford et al., 1990). Avoiding mixing of replacement cattle without screening for brucellosis and promoting the use self-contained units instead of shared facilities could help control brucellosis. In case of abortion, the aborted fetus must be properly disposed under biosafety precautions. Avoid burying infected fetuses because dogs and other wild animals may dig them up and disseminate the disease. Any entrance where the animals are located must use step in tanks on the floor filled with disinfectant.

Training of farmers

Training of the livestock farmers on the effective implementation of sanitary and hygienic livestock management practice following abortion helps reduce spreading the disease amongst animals as well as to the humans. Education of the farmers and animal care workers on the basic hygiene and sanitary procedures and techniques as well as practical demonstration on the use of disinfection and personal protection methods are important (Sammartino et al., 2005).

Use of vaccines

The use of vaccines is one of the important measures for prevention and control of brucellosis. In areas with endemic brucellosis only vaccination will control brucellosis. *Brucella* vaccines in use for livestock are the *B. melitensis* Rev 1, live *B. abortus* strain 19, and *B. abortus* strain RB51. The Rev 1 vaccine is a modified live *B. melitensis* vaccine used in small ruminants between the ages of three and four months that confers immunity for three to five years

(Blasco and Molina-Flores, 2011). Use of strain RB51 vaccine in cattle could be a good choice for control of brucellosis in Bangladesh. The use of strain RB51 has been shown to help prevent, control and eradicate cattle brucellosis in the countries where it has been adapted (Luna-Martinez and Mejia-Teran, 2002; Rahman and Baek, 2008b; Rahman 2011d).

Test and slaughter

In order to be a cost-effective disease control measure, test and slaughter is best implemented in areas where there is a less than two percent prevalence of brucellosis in the flocks and herds (Corbel, 2006). In developing countries, test and slaughter can be difficult to carry out due to enormous cost involved in the indemnification paid to the farmers for slaughtered animals (Blasco and Molina-Flores, 2011).

Enhanced biomedical research

Biomedical research focusing on epidemiology, isolation and characterization of field isolates, development of the best diagnostic method and more effective vaccines against brucellosis in non-bovine species should be undertaken. To date, only one published reports is available on the characterization of the *Brucella* isolates of animals in Bangladesh at the species level (Rahman et al., 2013a).

Government commitment

Regulations and adequate monetary support (political will) from the local and national government organizations strengthen the collaboration among farmers, veterinarians and regulators that are essential for effective implementtation of a country-wide brucellosis control and eradication program.

CONCLUSIONS

Brucellosis is considered as a neglected bacterial zoonotic disease in Bangladesh and it is present in the domesticcated animal species and humans in surveyed regions of Bangladesh. The differences in seroprevalence of brucellosis in the study areas may be linked to ecological factors, differences of animal's density and husbandry practices and type of serological tests. The variation of prevalence of brucellosis between animals on farms and domestic holding are likely to be attributed to certain risk factors such as cattle management practices, population dynamics and biological features (for example, host immunity) that largely influence the epidemiology of *Brucella* spp. Prevalence of brucellosis is higher in sexually mature and pregnant animals, in female than male, in animals with history of abortion than without history of abortion. A detailed and statistically valid surveillance study of brucellosis in high-risk group of people and domesticated animals throughout the country is necessary to know actual disease burden. Public health education for target groups of people, understanding the risk factors of brucellosis, hygienic animal management practice (biosafety), early diagnosis, collaboration among veterinarians, medical doctors and farmers and vaccination of animals are necessary for control of brucellosis in Bangladesh.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Evaluation of *Streptomyces* as probiotics against vibriosis and health management of prawn larvae *Macrobrachium rosenbergii*

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Nearly forty-five isolates of *Streptomyces* were found to be associated with 16 different seaweeds. Of these, only six strains that antagonized *Vibrio vulnificus* were selected to study the probiotic efficiency. Six strains were characterized based on the phenotypic characteristics. The toxicity of different Vibrio species (*V. harveyji*, *V. parahemolyticus*, *V. alginolyticus*, *V. vulnificus)* towards prawn larvae were studied, among them, *V.vulnificus* was more pathogenic and mass mortality (88%) was observed within three days. Hence, *Streptomyces* that antagonized *V. vulnificus* with maximum inhibition zone was selected for the probiotic study. *In vitro* and *in vivo* evaluation of probiotic efficacy of six selected *Streptomyces* were made using prawn larvae *Macrobrachium rosenbergii* challenged with *V. vulnificus* at 10^5Cfu/ml. The best growth-rate, reduced mortality, highest glucose and protein-level, reduced cholesterol level and increased enzymatic activities were noticed with *Streptomyces* AQB.SKKU8, AQB.SKKU18, AQB.SKKU25 treated groups. It is recommended to use strain AQB.SKKU 8, Strain AQB.SKKU 18, strain AQB.SKKU 25, and strain AQB.SKKU37, as probiotics *in-vivo*, while strain AQB.SKKU10, and strain AQB.SKKU20 showed probiotic effects *in-vitro* only. The results of the present investigation reveal that the seaweed associated *Streptomyces* were found to be a promising source of probiotic and biocontrol agents against vibriosis disease in aquaculture.

Key words: *Streptomyces* spp., physiological activities, biocontrol agent, vibriosis, disease resistance.

INTRODUCTION

Probiotics are live microbes, which confer health benefit to the host and have been used in aquaculture as biocontrol agents against various fish and shellfish diseases. Bacterial disease, especially vibriosis is a major disease problem in shrimp aquaculture causing high mortality, and severe economic loss in all producing countries. The major species causing vibriosis in shrimp are *Vibrio vulnificus*, *Vibrio anguillarum*, *Vibrio harveyji*,

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Vibrio alginolyticus and *Vibrio parahaemolyticus* (Goarant et al., 1999). Lin et al. (1989) showed that seven *Vibrio* species associated with vibriosis in silver sea bream *(Sparus sarba)* and of these species*, V. alginolyticus, V. vulnificus,* and *V. parahaemolyticus* are dominant. Among the vibrios*,* much attention focuses towards *V. vulnificus* during the last decade for its role both in human (Hoyer et al., 1995) and in fish pathogen (Tison et al., 1982; Biosca et al., 1991; Arias et al., 1997). Several strategies have been proposed to control vibriosis. For instance, the use of antimicrobial in disease prevention and growth promotion can bring about the emergence of drug-resistant microorganism and leave antibiotic residues in the prawn and the environment. Moreover, the chemotherapy may kill or inhibit intestinal microflora in the digestive tract, which are beneficial to the host (Sugita et al., 1999). Therefore, developing alternative technology for the health management of prawn larvae in the hatchery from vibriosis is the use of probiotic technology, which is an application of microbial ecology (Moriarty, 1999). Probiotics are applied to the feed or added to the culture tank or in a pond as a biocontrol agent against infection by pathogenic Vibrios. This approach to disease control is not much used in the prawn larval culture. Most proposed probiotics are Lactic acid bacteria, *Bacillus* strains and *Pseudomonas* strains (Verschuere et al., 2000), but few studies on probiotics consisting of *Streptomyces* have been reported as biocontrol agents against vibriosis disease.

Many studies on probiotics in aquaculture used in *vitro* models of specific bacteria as antagonists of pathogens (You et al., 2007; Selvakumar and Dhevendaran, 2010; Ngo Thi Tuong et al., 2011). Other studies have focused on growth promotion and improvement of water quality by probiotic *Streptomyces* supplements (Das et al., 2006; Selvakumar and Dhevendaran, 2010). There are reports on the *Streptomyces* used as single cell protein feed on the growth of juveniles of *Macrobrachium* (Manju and Dhevendaran, 2002). *Streptomyces* culture extracted using fermentation media also used to evaluate the survival of black tiger shrimp against White spot syndrome virus (WSSV) infection has shown significant survival. *Streptomyces* cell mass was supplemented with feed at 1% dosage to black tiger shrimp for 15days in three treatments with two treatments of commercial probiotic that resulted better growth, survival and mortality of *P. monodon* after exposure to virulent *V. harveyji* at 10^7 CFU ml^{-1}. The significant reduction in the percentage mortality of *Artemia* was observed after 24, 48 and 72 h immersion in different concentrations of *Streptomyces* cell mass (Surajit et al., 2006). Jayasudha et al. (2011) studied *in vitro* inhibitory activity of ethyl acetate extract of actinobacteria, which showed 33.56% mortality and did notice any relative percentage inhibition in the petroleum ether extract. Giant freshwater prawn *(Macrobrachium rosenbergii* de Man, 1879) or scampi used in this study is an important commercial candidate species due to increased protein demand in domestic and export markets. Considering above facts, the present study attempted to develop the marine *streptomycetes* into probiotic to control vibriosis disease in aquaculture. The *Streptomyces* was isolated from the seaweeds of marine coast and it's *in vitro* and *in vivo* probiotic potential were tested against *Vibrio* spp., especially against *Vibrio vulnificus* have been surveyed in this study. In addition, an attempt was made for the first time to study the efficacy of *Streptomyces* as probiotics on promoting physiological activities of prawn, *M. rosenbergii* thereby to improve the health.

MATERIALS AND METHODS

Isolation of *Streptomyces* from seaweeds

The Seaweed samples were collected during September 2006 from Muttom coast, Tamil Nadu, India (8° 7' 15" N: 77° 1'E) at monthly interval by random sampling method. One-gram mantle of live specimen was weighed, washed with distilled water and treated with 0.5% phenol for 5 min to eliminate the bacterial and fungal colonies present as contaminants. After the distilled water wash, the mantle was macerated and aliquots of 1 ml was serially diluted with sterile seawater and plated each dilution on three different selective media. The total number of *Streptomyces* colonies on the plates was enumerated (Okazaki and Okami, 1976; Lakshmanaperumalsamy, 1978). They were incubated at room temperature (28±2°C) for seven days. The colonies were counted and expressed as CFU/gm dry wt. of visceral mass. The isolated strains were stored in Glycerol asparagine agar (ISP5) medium as agar slant cultures at 28±2°C (Shirling and Gottlieb, 1966).

Determination of *in vitro* probiotic activity

The pure isolates were examined for its antagonistic effects against the four shrimp pathogenic *Vibrio* species (*V.harveyji*, *V.alginolyticus*, *V.parahaemolyticus* and *V. vulnificus*). The *in vitro* probiotic activity was assessed using double layer method. The *Streptomyces* isolates were inoculated on petridish containing 15ml GsB agar medium (soluble starch 5 g, soybean meal 5 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 µM, seawater to 1 L) and incubated at 28±2°C for 3 days. The chloroform vapour was applied over the colonies. Sloppy (0.75% TCBS) agar containing *Vibrio* spp. was seeded onto the basal layer of GsB agar plate. The zone of inhibition was measured after incubation at 28°±2C for 24 h.

Identification of probiotic bacteria

Probiotic bacterial strain, *Streptomycetes* used in the study was characterized according to traditional morphological, cultural, biochemical characteristics as described in Shrilling and Gottlieb (1966).

Morphological characters

Aerial mass color determination

The cultures were inoculated on starch-casein agar and glycerol asparagine agar, incubated at 28±2°C for seven days and the

Table 1. Percentage composition of probiotic and control feed.

Ingredient	Control feed (w / %)	Probiotic feed (w/ %)
Prawn Meal	25	25
Fish Meal	15	15
Soybean Meal	10	10
Groundnut oil cake	15	15
Wheat flour	15	15
Tapioca	13	13
Vitamins	1	1
Minerals	1.5	1.5
Cholesterol	0.5	0.5
Streptomyces biomass	0	7×10^5 cells/g/ml
Oil	4	4
Total	100	100

mature sporulating aerial mycelium was observed for their colorations. The cultures were classified into white, grey, red, green, blue, yellow and violet series, depending on the aerial mycelial color.

Micromorphological characteristics

The *Streptomyces* isolates was streaked on Petri dish containing starch-casein agar medium and incubated at room temperature (28±2ºC) for 7-10 days. The growth on the surface of the medium was observed under Phase contrast microscope (magnification 400X). Depending upon the number of spores and sporulating pattern, strains are categorized into different species.

Substrate mycelium color

The color on the reverse side of *Streptomyces* colonies was observed using starch-casein agar and glycerol asparagine agar. The strains are classified into pale yellow, olive, yellowish brown or cream depending on the substrate mycelial color.

Soluble pigments

The isolates were cultured on salts starch-casein agar and observed for soluble colors other than melanoids (brown or black). The strains were divided into two groups viz: positive (+) and negative based on their ability to produce soluble pigments. The color was recorded as red, orange, green, yellow, blue and violet.

Melanin production

The cultures were streaked on peptone-iron agar slants supplemented with 0.1% yeast extract and incubated at 28±2□C for 48 h. Culture-forming greenish-brown to brown and then to black diffusible pigments were recorded as positive (+) melanoid production, whereas the absence of these colors was recorded as negative (-) for melanoid production.

Carbon utilization

The *Streptomyces* colonies were inoculated into10 ml of basal minerals to which sterilized carbon sources (Xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose and glucose) wer added to a final concentration of 1%. The tubes were incubated at 28□C for seven days. The growth was observed and categorized into positive and negative. The growth was compared with two controls; growth without carbon source was used as negative control and growth on D-glucose was used as positive control.

Species level identification of *Streptomyces* isolates

Streptomyces isolates were identified at the species level by comparing the phenotypic properties with the representative species found in the key of Nonomura (1974) and Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

Preparation of probiotic bacterial cell suspension

Fresh cultures of each of the selected probionts were inoculated into 100 ml of Glycerol asparagine broth and incubated at 28±2□C for 4 days in a shaker, and was centrifuged at 4□C for 15b min at 10,000 rpm. The supernatant was discarded and the pellet was resuspended in 5ml of PBS (Phosphate buffer saline). Five millilitres of fish oil was added to each fraction and mixed thoroughly in a vortex mixture. The bacterial cell suspension was mixed with the formulated feed ingredients.

Preparation of probiotic feed

Seven different feeds were prepared, namely the probiotic (6) and control feed (without bacterial suspension). One hundred grams compound feed were prepared (Hardy, 1980) as per the formulation given in Table 1. The dough was prepared by adding the required amount of water of these ingredients; it was sterilized by steam (autoclave at 121°C for 15 min) and incorporated with a commercial vitamin and mineral mix at 2.5 (v/w) and pelletized using hand pelletizer to obtain 1 mm pellets. The pellets were initially sun dried and further dried in an oven at 60±5°C for 12 h. They were manually broken into smaller bits and stored in airtight sterile polypropylene containers at room temperature.

The probiotic feeds (PS_1, PS_2, PS_3, PS_4, PS_5 and PS_6) were prepared by mixing each probiotic cell suspension of the compound feed in order to obtain evenly coated pellets of each feed containing 7-×10^5cells g-1of diet. The control feed contains only PBS and oil. The feeds were stored in sterile airtight containers at 4°C. The viability of the bacterial cells was determined periodically.

Table 2. Proximate composition of formulated probiotic and control feeds.

Formulated feeds	Proximate composition (% dry matter basis)					
	Moisture	Crude protein	Crude fat	Ash	Crude fibre	Nitrogen free Extract (NFE)
Control	6.95	37.84	5.5	12.14	1.6	35.96
PS_1	6.73	47.91	3.9	10.14	3.78	31.12
PS_2	6.78	47.84	3.98	10.3	3.56	30.83
PS_3	6.74	47.96	3.86	10	3.8	31.1
PS_4	6.76	47.88	3.92	10.28	3.58	31.52
PS_5	6.68	48.12	3.8	9.72	3.87	31.02
PS_6	6.7	48.08	3.82	9.74	3.85	31.06

DC, basal diet without bacteria (Control feed); PS_1,basal diet containing probiotic strain AQB.SKKU8; PS2 , basal diet containing probiotic strain AQB.SKKU10; PS3 , basal diet containing probiotic strain AQB.SKKU18; PS_4 , basal diet containing probiotic strain AQB.SKKU20; PS_5, basal diet containing probiotic strain AQB.SKKU25; PS_6, basal diet containing probiotic strain AQB.SKKU37.

Proximate chemical analysis of formulated feeds

The proximate composition of the feed was analyzed by moisture, crude protein, crude fat, crude fibre, ash and nitrogen-free extract according to the standard methods of AOAC (1990). The proximate compositions of the formulated feeds are displayed in Table 2.

In-vivo evaluation probionts

The isolated probiotic bacteria tested for its *in vivo* role against pathogenic *V. vulnificus* was evaluated by using 50 healthy *M. rosenbergii* larvae to each group. The larvae were acclimatized for two weeks in indoor tanks. The shrimp larvae were divided into seven equal groups with three replicates per each group. Six groups (1-6) were fed with probiotic diet and a group (7) was fed with control diet for two weeks. The percentage survival was noticed prior to challenge. Six experimental groups and control group were further challenged with *V. vulnificus* to the rearing water to get 10^5 CFU/ml. All groups were kept under observation for more than two weeks and the mortality was recorded. For microbiological analysis, water sample was taken after 30 days of the experiment. Total *Vibrio* count during the rearing was recorded using Thiosulphate citrate agar medium. During this period, no water exchange was provided and the water quality parameters were maintained as recommended by the Correia et al. (2000).

Physiological analyses

The healthy post-larvae *M. rosenbergii* used for physiological studies were maintained in indoor tanks. At the end of the experimental period, haemolymph was collected from the animal. Plasma was obtained by centrifugation of heamolymph at 3000 rpm for 15 min and non-haemolyzed plasma was stored in deep freezer for further biochemical analysis.

The heamolymph glucose, protein and cholesterol were determined calorimetrically by using available commercial kits purchased from Erba Mannheim Diagnostic Company, TRANSASIA Biomedical Ltd. The Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined calorimetrically according to Reitman's and Frankel (1957).

Statistical analysis

The obtained results were analyzed statistically using One-way analysis of variance (ANOVA). Turkey HSP multiple comparison test was used to evaluate the mean ratio among the different treatments at 5% probability level. All the statistical analyses were performed using the SAS program (2005).

RESULTS

Isolation and enumeration of streptomycetes from seaweeds

The occurrence, distribution and identification of different kinds of seaweeds were surveyed in the Muttom (marine) coast during the study period. During the study, 16 different types of seaweeds were obtained. Enumeration of *Streptomyces* associated with seaweeds also exhibits *Sargassum weight* and *Enteromorpha intestinalis* are being the source of the highest number of pigment producing strains. The maximum *streptomycetes* population recorded in glycerol asparagine agar in the month of October followed by June (73 $\times 10^2$cfu/ml) and February (68 $\times 10^2$cfu/ml). The minimum streptomycetes population was recorded in the month of November ($5X10^2$cfu/ml) followed by the month of July (14 $\times 10^2$cfu/ml); was recorded on the coast (Figure 1).

The *invitro* probiotic activity

In the present study, a total of 45 *streptomycetes* colonies were isolated from seaweeds of marine coast and tested for its *in vitro* probiotic activity against four pathogenic *Vibrio* spp. It was found that out of the 45 isolates, only 25 isolates of *Streptomyces* showed antagonistic activity against any one of four tested *Vibrio* spp. (*V. harveyji*, *V. alginolyticus*, *V. parahemolyticus* and *V. vulnificus*). Between 25 probiotic isolate, 23 (92%) isolates inhibited *V. harveyji*, 18 (72%) isolates were active against *V. alginolyticus*, 13 (52%) of isolates were antagonistic to *V. vulnificus*, and 15 (60%) isolates inhibited *V. parahaemolyticus* (Table 3).

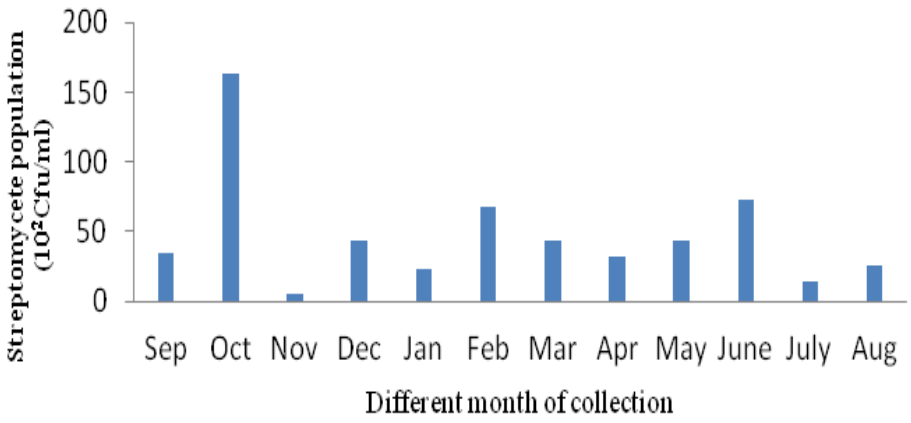

Figure 1. Streptomycetes population recorded in the different months.

Table 3 . Number of *Streptomyces* isolates antagonized tested pathogenic strains.

Parameter	Tested pathogenic strains			
	V. harveyji	***V. alginolyticus***	***V. vulnificus***	***V. parahaemolyticus***
No. of *Streptomyces* species against *Vibrio* spp.	23/25	18/25	13/25	15/25
Percentage	92	72	52	60

Identification of *streptomycetes* from seaweeds

The isolates of *streptomycetes* were identified using pheno-typic characteristics using Nonomura Key and Bergey's manual of determinative bacteriology (1974). The isolates were identified as closely related to *Streptomyces coelicolor (AQB.SKKU 8), Streptomyces autotrophicus (AQB.SKKU 10), Streptomyces pedanensis (AQB.SKKU 18), Streptomyces deccanensis (AQB.SKKU 20), Streptomyces vinaceus (AQB.SKKU 25)* and *Streptomyces Nov spp. (AQB.SKKU 37).* Their morphology, pigmentation and carbon utilization characteristics were depicted in Table 4.

In vivo effect of the isolated probiotics

The rate of survival was 100% in pre-challenged larvae fed with probiotic feed, compared to the control (85.42 ± 3.60). The strain *AQB.SKKU.8* (PS_1), strain *AQB.SKKU.18* (PS_3), strain *AQB.SKKU.25* (PS_5) and strain *AQB.SKKU.37* (PS_6), of the probiotics were harmless to the shrimp *M. rosenbergii* larvae, as there were no clinical signs and reduced mortality was noticed than control. The percentage mortality of 0-18% was observed in probiotic treated groups than control (Table 5) which showed a mortality rate of 79.2%. The total *Vibrio* count determined subsequent to the addition of inoculum was significantly lesser ($p < 0.05$) than that of the control.

Physiological parameters

The glucose and protein level was significantly increased in the heamolymph of prawn fed with probiotic diet PS_1 (containing strain AQB.SKKU8), PS_5 (containing strain AQB.SKKU25) and PS_6 (containing strain AQB.SKKU37) ($P>0.5$) compared to the control diet (feed devoid of Streptomyces). The glucose level was lower in control and PS_2 diet (containing strain AQB.SKKU10), while the low protein levels were obtained in the PS_2 (containing strain AQB.SKKU10) and PS_4 diet (containing strain AQB.SKKU20). The data obtained are given in Table 6.

The AST and ALT activities in plasma of *M. rosenbergii* increased in the probiotic treated groups such as PS_1, PS_3 and PS_5 than in the control. The lowest values were obtained in the control, PS_2 and PS_4 diet. The average levels of plasma AST and ALT activities with *M. rosenbergii* in the control group was 38.73±6.28 and 19.25±2.18 IU/L respectively and the results are presented in Table 6.

Growth performance

Experiments were performed to study the effect of probiotic isolates on growth enhancement of freshwater prawn, *Macrobrachium rosenbergii*. During the 30 days feeding trial, the prawn consumed well and acceptability of all diets was similar. Among the probionts, PS_5 diet produced elevated values in weight gain followed by PS_6

Table 4. Phenotypic characteristics of *Streptomyces* spp. Isolated from seaweeds.

Strain number	Seaweed source	Spore chain morphology	Spore surface	Aerial mass color	Reverse side color	Melanoid Pigment	Soluble Pigment	Carbon utilization								
								Glu	Ara	Xyl	Ino	Man	Fru	Rha	Suc	Raf
AQB.SKKU8	*Gracillaria corticata*	RF	Smooth	Yellow/ Green	Brown /lavender	-	-	+	+	+	-	+	+	±	±	-
AQB.SKKU10	*Chnoospora minima*	RF	Smooth	Pale yellow	ND/ cream	-	-	+	+	+	+	+	+	+	+	-
AQB.SKKU18	*Sargassum weightii*	RF	Smooth	White	Brown	-	-	+	+	+	±	+	-	±	+	-
AQB.SKKU20	*Spyridia hypnoides*	SC	Hairy	Grey	Yellow	+	+	+	+	+	+	+	+	+	+	+
AQB.SKKU25	*Enteromorpha intestinalis*	RF	Smooth	Yellowish brown	Reddish brown	-	-	+	-	+	-	+	+	-	+	-
AQB.SKKU37	*Hypnea valentiae*	RF	Smooth	Bluish green	Reddish Brown/red	-	-	+	±	+/	+	+/	+/	+	+	±

RF, Rectiflexibles; SC, Straight chains; ND-not distinctive; Glc, D-glucose; Ara, L-arabinose, Fru,-D-fructose; Ino, iinositol; Ma, D-Mannitol; Raf, raffinose, Rha, L-rhamnose. Su, sucrose; Xyl, D-xylose, "+": well utilized;" +/_": poorly utilized; "_", not utilized; NA, not available.

Table 5. Post- challenge percentage mortality of shrimp larvae *M. rosenbergii* fed with control and probiotic feed.

Parameter	DC	PS_1	PS_2	PS_3	PS_4	PS_5	PS_6
%Mortality after 7days of infection (Mean± S.D)	64.6±3.60	14.2±0.0^{a}	54.2±3.60	18.8± 0.0^{a}	31.3 ±6.3	0 ± 0	10.4±3.6^{a}
%Mortality after 2weeks of infection (Mean± S.D)	79.2 ± 3.6	14.2±0.0^{a}	58.3 ± 7.2	18.8± 0.0^{a}	33.3 ±3.6	2.1±3.6^{b}	10.4±3.6^{ab}

Means having the same letter in the same row are not significant ($P < 0.05$). DC, basal diet without bacteria (Control feed); PS_1,basal diet containing probiotic strain AQB.SKKU8; PS2, basal diet containing probiotic strain AQB.SKKU10; PS3, basal diet containing probiotic strain AQB.SKKU18; PS_4, basal diet containing probiotic strain AQB.SKKU20; PS_5, basal diet containing probiotic strain AQB.SKKU25; PS_6, basal diet containing probiotic strain AQB.SKKU37nov Sp.

Table 6. Physiological parameters of shrimp *M. rosenbergii* fed with different probiotic and control feeds.

Parameter	DC (control)	PS_1	PS_2	PS_3	PS_4	PS_5	PS_6
Glucose	8.75±2.75^{a}	20±5.88^{b}	5±1.82^{a}	12±1.41^{ab}	11±0.81^{ab}	29.75±0.70^{c}	17.75±0.95^{b}
Protein	2.7±2.63a	7.4±0.08b	4.39±0.36a	6.89±0.36ab	1.25±0.18a	10.9±0.50c	7.69±0.69b
Cholesterol	17.5±1.29a	4±0.3b	15±1.81a	7±0.81c	9±0.81d	2 ±1.41b	3±0.81b
AST (IU L^{-1})	38.75±6.29^{a}	42 ±6.48^{a}	8±2.16^{c}	39.25±2.06^{d}	37.75±1.70^{a}	56.25±2.62^{e}	46±6.48^{f}
ALT (IU L^{-1})	19.25±2.18^{a}	23±2.44^{a}	12±2.82^{a}	22.75±2.44^{a}	19±1.41^{a}	30.5±3.69^{a}	32.5±6.24^{a}

Means having the same letter in the same row are not significant ($P < 0.05$). DC, basal diet without bacteria (Control feed); PS_1, basal diet containing probiotic strain AQB.SKKU8; a PS2, basal diet containing probiotic strain AQB.SKKU10; PS_3, basal diet containing probiotic strain AQB.SKKU18; PS_4, basal diet containing probiotic strain AQB.SKKU20; PS_5, basal diet containing probiotic strain AQB.SKKU25; PS_6, basal diet containing probiotic strain AQB.SKKU37, AST, aspartate aminotransferase; ALT, alanine aminotransferase; (IU L^{-1}) , International Unit/Liter.

diet compared to the control. The lowest growth rate was obtained with the control diet, which exhibited the highest mortality (Table 7).

DISCUSSION

The phenotypic characteristics of the isolated strains from

Table 7. Growth performance of *M. rosenbergii* fed with control and putative probionts.

Parameter	Control	(PS_1)	(PS_3)	(PS_5)	(PS_6)
Average Initial length (cm)	43.70±0.01[a]	47.73±0.03[a]	43.72±0.01[a]	43.74±0.02[a]	43.70±0.02[a]
Average Initial weight (g)	25.57±0.01[a]	25.59±0.03[a]	25.57±0.02[a]	25.26±0.02[a]	25.58±0.01[a]
Average Final length (cm)	47.03±0.01[ab]	47.74±0.01[ab]	47.38±0.02[ab]	53.0±0.01[ab]	52.70±0.03[ab]
Average final weight (g)	28.4±0.02	33.35±0.06[a]	32.44±0.03[b]	33.75±0.04[ab]	35.58±0.02
Specific growth rate (SGR)	0.094±0.06	0.26±0.02[a]	0.229±0.03[b]	0.273±0.02[ab]	0.27±0.04
Length gain	7.62±0.02[a]	9.31±0.01[b]	8.47±0.02[ab]	21.35±0.06[ab]	20.59±0.04
Weight gain	11.07±0.03	30.50±0.02[a]	26.87±0.01[a]	32.04±0.02[ab]	31.27±0.01[b]

Means having the same letter in the same row are not significant ($P < 0.05$). DC, basal diet without bacteria (Control feed); PS_1,basal diet containing probiotic strain AQB.SKKU8; PS2 , basal diet containing probiotic strain AQB.SKKU10; PS3 , basal diet containing probiotic strain AQB.SKKU18; PS_4, basal diet containing probiotic strain AQB.SKKU20; PS_5, basal diet containing probiotic strain AQB.SKKU25; PS_6, basal diet containing probiotic strain AQB.SKKU37, g,gram; cm,centimeter; SGR,Specific growth rate; S.D , Standard deviation.

the seaweeds are pointed out in different *Streptomyces* species; further molecular characterization is needed for their taxonomic position. *Streptomyces* isolated from seaweed exhibited maximum antagonism of 6.1 against *V. vulnificus in vitro*. Vanajakumar (1981), Gomathinayagam (1995) and Mathew et al. (1994) were observed differential antagonistic property of microbes from different source against test pathogen. Gildberg et al. (1995) and Nogami and Maeda (1992) reported *in vitro* antagonism test are the common way to screen the candidate probiotics against finfish and shellfish pathogens.

In the present study, higher percentage survival with no external disease manifestations was detected in probionts fed *M. rosenbergii* juveniles challenged with *V. vulnificus* infections, whereas 79.2% mortality was obtained in animals maintained on feed devoid of *Streptomyces* probiotic. Earlier studies of Sridevi and Dhevendaran (2007) stated that *V. vulnificus* cause mass mortality in *M. rosenbergii* during challenge with four different *Vibrio* spp. The studies of Garriques and Arevelo (1995) well correlated with the present findings and recommended the use of probiotics for increasing the disease resistance of farm animals. Limited studies were carried on the aspect of *in vivo* probiotic effect of *Streptomyces* against *Vibrio* species, especially against *V. vulnificus*. The present study is also in agreement with those of Austin et al. (1995), who challenged Atlantic salmon with *V. alginolyticus*, feed with a probiotic feed for 7 days, and reported 66% mortality in the control group as compared to 24% mortality in the probiotic fed growth. The larvae fed with probiotic *Bacillus* survived for 100 days (100%) challenged with *V. harveyji* in comparison to only 26% survival in the group not fed with probiotic (Rengpipat et al., 1998). The result of the present study also clearly demonstrated that *Streptomyces* act as potent biocontrol agent, which destroys the *Vibrio* spp. thereby improving the survival during the challenge**.**

The biochemical analyses often provide vital information on health-assessment and management of culture fish (Blaxhall, 1972; Cnaai et al., 2004). The present study reveals that haemolymph protein and glucose contents were enhanced by the supplement of *Streptomyces* probionts. Similar results were obtained in White shrimp (*Litopenaeus vannamei*) fed six different formulations of medicinal herbs (MH) and *Bacillus* in a feeding trial (Ming-Chaoyu et al., 2008). The results show similar trends to those reported in *O. niloticus* (Mohamed, 2007; Mehrim, 2008). Contrary results were observed with Abd El-Rhman et al. (2009), who found the various biochemical parameters reduced in fish diets supple-mented with *Pseudomonas* species. This mean, the species might be changed to pathogenic-version. Abdelhamid et al. (2009) also reported that the low total protein in fish blood while treated with probiotics due to insufficient amount of protein in the diet might lead to low protein of blood. This is in the case of starvation, because catfish is generally fed once daily, which commonly called starvetion.

In the present study, plasma cholesterol content decreased significantly in the treated group than the control. These results correlated with the findings of Lin et al. (1989), who found that probiotics and therapeutic properties of probiotic control the serum cholesterol levels. Maan and Sproeig (1974) also reported that drinking yogurt fermented with wild strains of *Lactobacillus* species reduced the values of blood cholesterol. Nutritional status is considered as one of the important factors that determine the ability of animals to withstand infections. Hence, nutritional status is considered a good health indicator of shrimp (Merchie et al., 1998; Bachere, 2000). These findings mean that the addition of probiotic *Streptomyces* isolates led to significant increases in the plasma protein, which indicates the improvement of the nutritional value of the diet. The results of the present study also demonstrated that blood glucose and cholesterol together with blood proteins are good indicators of nutritional health.

The present study indicated that the AST, ALT activities in plasma of *M. rosenbergii* were significantly increased In shrimp fed with diets containing strain AQB.SKKU8

and strain AQB.SKKU18 (PS_3 and PS_4). Tahovonen and Avikainen (1987) and Austin et al. (1995), who found probiotic bacteria, improve the physiological state of the crab larvae (*Portunus trituberculatus*). Khattab et al. (2004) and Palikova et al. (2004), who indicated a decrease of enzyme activities (AST, ALT and LDH) in *Cyprinus carpio* after exposure to extract of Cyanobacterium, observed the contrast results. The enzymatic activity was decreased in the control group due to the severe damage of some organs, liver, spleen, muscle and kidney (Cruz et al., 1989). The present study concluded that probiotic *Streptomyces* might improve the physiological state of the *M. rosenbergii* by serving as a nutrient source during its growth.

During the probiotic feeding, the final weight and survival rate were increased among prawn fed with diet containing PS_5 (strain AQB.SKKU25), so, it may be considered as a growth promoter in confined systems. These results are well correlated with those of Manju and Dhevendaran (1997), they reported that the marine streptomycetes as single cell protein was incorporated into the artificial diet increased growth, food conversion efficiency and protein increment of the juvenile prawn, *Macrobrachium idella* and fish, *Oreochromus mossombicus*. The present study also supported by Abdelhamid et al. (2009) noted that the tested probiotic reflected better results than the control. The growth and metabolism of fish were associated with the microflora present in the alimentary tract and the use of artificial feed can meet the nutritional requirements of fish to a considerable extent (Maya et al., 1990). The present study was supported by those of Irianto and Austin (2002). *Streptomyces* used in this study as probiotics, is also well known for producing many secondary metabolites. The present study concluded that probiotics might stimulate the appetite and improve nutrition by the production of secondary metabolites such as pigments, vitamins, amino acids, peptides, and detoxification of compounds in the diet and by the breakdown of indigestible components. Probiotics are considered the best to promote growth as well as health of organisms by inhibiting the proliferation of pathogenic bacteria in the same habitat (Bogut et al., 2000). This has led to a new strategy for prevention of disease outbreak and fish health management in aquaculture operations.

Conclusion

The investigations in the form of challenge studies of these probionts in reared animals with common shrimp pathogen *V.vulnificus* clearly demonstrated that antagonistic *Streptomyces* as a potent probiotic against vibrios, especially against *V. vulnificus* and can be used for other aquaculture practices. To the best of our knowledge, this is the first report on the combined probiotic and biocontrol role against *Vibrio vulnificus*. This study proved that the *Streptomyces* probiotics improved the survival, growth, physiological activities and disease resistance against vibriosis. All the beneficial influence of probiotics discussed here indicates that *Streptomyces* probiont are going to play an important role in the near future, especially against frequently causing disease.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of two T4-like bacteriophages against pathogenic *Escherichia coli* of piglet

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This study, has described the isolation of two T4-like JH11 and JH14 phages. All bacteriophages produced clear plaques in double Luria Bertani (LB) agar, but JH11 produced the larger plaques. These phages lysed *Escherichia coli* O141: F18 (ETEC), they have icosahedral heads, necks and contractile tails, and belonged to the *Myoviridae* family. The genomes of these phages were a strand DNA, predicted to be larger than 2 kb. Titer of the two phages were unaffected by exposure to pH 5 - 9 for 16 h. Among the phages, JH11 and JH14 were resistance to enterotoxigenic *E. coli* O141: F18 (ETEC). Both phages contain three abundant protein bands with approximate molecular sizes of 80, 75 and 50 kDa for JH11, and 50, 38, and 36 kDa for JH14. According to the blast results, the phage JH11 random clones are in close relation with phage T4 gene 23 for major capsid protein MVSS (98%), ECML-134 (98%), T6(97%) and T4 (96% identity) identity. Phage JH14 random clones are in close relation with HX01 (96%), CEV1 (95%) and T4 (93%) identity. The two strains belong to T4-like phage genus. Gene of JH11 and JH14 were (93%) identical.

Key words: Bacteriophage, enterotoxigenic, *Escherichia coli,* T4-like phage.

INTRODUCTION

Bacteriophages or phages are enteric viruses that can replicate only inside susceptible bacteria, There are diverse forms of organisms in the world and exert a major influence on the microbial world (Hendrix et al., 1999). While phages have been proposed for the treatment of bacterial diseases (Barrow et al., 1998), the nature of phage-host interactions, poor understanding of mechanisms of bacterial pathogenesis and introduction of antibiotics have hampered the investigations of their role in therapy (Soothill, 1992). Enterotoxigenic *Escherichia*

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coli (ETEC) are a cause of porcine diarrhea, that may occur sporadically or as outbreaks leading to substantial economic losses (Amezcua et al., 2002). Antibiotics have been commonly used in the treatment of infectious diseases, but their wide spread and improper use has led to antibiotic resistance in porcine colibacillosis (Lu and Koeris, 2011). Nevertheless, the potential use of phages as therapeutic agents in controlling human and animal disease has been recognized (Carey et al., 2006). Recently, there have been report on the isolation and application of phage in the treatment of animals with resistant *E. coli* infections (Jamalludeen et al., 2007). Bacteriophages were used to prevent and treat O149 colibacillosis in pig as also been explored by Jamalludeen et al. (2009a).

The goal of the present study was to isolate phages with lytic activity against enterotoxigenic *E. coli* (ETEC) and to characterize them according to their morphology, sequence, genome size, etc.

MATERIALS AND METHODS

Bacteria strain

E. coli strains (n=31) were isolated from pig farms located in various regions of central Vietnam. All strains were kept at -80°C Luria broth (LB) with 20% glycerol for preservation.

Samples

The bacteriophages were isolated from 30 individual fecal samples, collected from two pig farms located in Jiangsu Province of China farms (during the period of October 2011 to Jun 2012).

Media and chemicals

Luria Bertani (LB) broth, LB agar, and LB top agar (soft agar) were prepared as described by Sambrook et al. (1989). Each litre of bacteriophage broth contained tryptone 10 g, yeast extracts 5 g, and NaCl 10 g, pH 7.5. TS buffer (8.5 g of NaCl and 1 g tryptone per litre). The following reagents were also used: RNase I, DNase I (Roche, Basel, Switzerland, cat no: 10104159001), proteinase K, and ethidium bromide (Invitrogen, Carlsbad, CA, USA).

Bacteriophage isolation and purification

LB broth was inoculated with mixture equal proportions of the VN11-O141:F18 ETEC and VN14-O141: F18 ETEC strains and incubated for 5 h at 37°C. The samples (5 g of fecal sample from pig farm in TS buffer) were centrifuged before filtering through a membrane filtered (0.45-µm membrane) to remove impurities and bacteria before being added to the host suspension. Twenty milliliters of LB broth, and 20 ml of a suspension of *E. coli* strains in broth culture (OD600 = 1.4) and 10 ml of sample were then added to the flask incubated at 37°C for 24 h in a shaker to enrich *E. coli* bacteriophages. After incubation, the culture was added NaCl 5% for 30 min at 4°C, centrifuged twice at 4,000 ×g for 15 min at 4°C, the supernatant was collected into a sterile flask and filtered through a sterile 0.45-µm membrane filter (Fisher Scientific). To detect the presence of phage in the filtrate, spot testing was performed as described previously by Kropinski et al. (2009). Phage preparations were obtained and stored at 4°C as described by Jamalludeen et al. (2009b).

Electron microscopy

Phage preparations were applied to a carbon film and fixed to a copper grid being negatively stained with phosphotungstic acid (PTA, 2% w/v). Electron micrographs were taken with an H_7650 (HITACHI, Japan) transmission electron microscope (TEM) operating at 80 kV. Both phage morphology and dimension (capsid diameter and tail length) are as described by Bai et al., (2013).

Host range analysis and stability assays

To investigate the sensitivity of *E. coli* strains to phage JH11 and JH14, 31 piglet isolates (VN1-31), 10 bovine isolates (JV1-10) and 10 chicken strains (LYT 15-25) were tested as described elsewhere (Jamalludeen et al., 2009b).

One-step growth curve and adsorption of bacteriophage

The phage adsorption assay was carried out according to Shlyapnikov et al. (1984, 1985)). 1 ml of phage in warmed water (37°C) suspension (1–3 $\times 10^5$ pfu/ml) was added in 9 ml of mid-log-phase bacterial culture and incubated at 37°C. At 2.5-min intervals, aliquots of 0.05 ml were removed and placed in chilled tubes containing 0.95 ml medium. Phage-cell complexes were removed by centrifugation (10,000 ×g, 10 min), and the titer of free un-adsorbed phage in the supernatant was then determined by the double-layer agar plate method (Kropinski et al., 2009). The one-step growth assay was carried out as described by Pajunen et al. (2002). In brief, 10 mL of the culture (containing 2×10^8 cfu/ml) was infected at a multiplicity of infection (MOI) of 0.1. Following incubation at 37°C for 2.5 min, the bacteria-phage mixture was diluted by 10^4-fold to abruptly end adsorption. Samples were taken after various inoculation times and centrifuged at 10,000 ×g for 2 min, and the phage titer of the supernatant was determined by the double-layer agar plate method (Kropinski et al., 2009).

Assessment of bacteriophage resistance to acidity and alkalinity

Resistance to acidity and alkalinity in suspensions of each of the two bacteriophages were evaluated by exposure to various pH con-ditions ranging from 1 to 11 and checked for survival over a 16-h period as described by Jamalludeen et al. (2007). 100-µl bacteriophage suspension 107 (pfu/ml) and 900 µl of normal saline, pH 7.2, was also incubated at 37°C for 16 h. After incubation, a 100-µl volume of the bacteriophage suspension was serially diluted 10-fold, mixed with 100 µl of host bacterium (10^8 cfu/ml) and incubated for 15 min at 37°C before being added to 4 ml of soft agar and spread over an LB plate. Titers of the surviving bacteriophage were determined by plaque assays with 10-fold dilutions using the soft agar overlay method.

Extraction of bacteriophage DNA

Bacteriophage DNA was extracted as described by Pickard (2009) and Sambrook et al. (1989). All bacteriophages were allowed to

completely lyse their host *E. coli* strains in a soft agar overlay. The overlay was added to SM buffer, and bacteriophages were allowed to diffuse into the buffer at 4°C for 3-4 h with gentle shaking as previously described. After the suspension was centrifuged at 4000 ×g for 15 min, the supernatant was collected. Solid NaCl was added to a final concentration of 1 M and dissolved by swirling. Following incubation on ice for 1 h, the suspension was centrifuged at 11,000 ×g for 10 min at 4°C (Beckman Coulter, J2-MC Centrifuge). The supernatant was collected, solid polyethylene glycol (PEG 8000) was added to a final concentration of 10% (w/v), and the mixture was stirred slowly at room temperature. After cooling in ice water and standing for 1 h on ice, the mixture was centrifuged at 14,000 ×g for 10 min at 4°C. The bacteriophage pellet was then resuspended in 1 mL of SM buffer. An equal volume of chloroform was added to the phage suspension and mixed by vortexing for 30 s. The phases were separated by centrifugation at 3000 ×g for 15 min at 4°C and the aqueous phase was recovered. Pancreatic DNase I and RNase I were added to a final concentration of 5 and 1 µg/mL, respectively, and allowed to digest substrates for 30 min at 37°C. EDTA, pH 8.0 was added to a final concentration of 20 mM. Proteinase K was added to a final concentration of 50 µg/mL, then sodium dodecyl sulfate (SDS, 10%) was added to a final concentration of 0.5% and the mixture was inverted several times prior to incubation at 56°C for 2 h. An equal volume of phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v) was mixed in the sample. The aqueous phase was collected after centrifugation at 10,000 ×g for 10 min and extracted with an equal volume of chloroform : isoamyl alcohol (24:1, v/v). Centrifugation was repeated and the aqueous phase was collected. Two volumes of ice-cold 95% ethanol were added and the mixture was kept at room temperature for 20 min. The precipitate was collected by centrifugation at 10,000 ×g for 10 min at 4°C and the pellet was washed with cold 70% ethanol. Following centrifugation at 10,000 ×g and 4°C for 30 min, the pellet was air dried and dissolved in 20-35 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

Agarose gel electrophoresis analysis

Genome sizes of undigested or digested bacteriophage were determined by electrophoresis (Sambrook and Russell, 2001). The bands were stained with ethidium bromide (EB) and compared against a λ phage DNA digested with Hind III marker and restriction enzyme *Dra* I before comparison by gel electrophoresis(Chakrabarti et al., 2000).

Protein of purified particles of phage

Bacteriophages particles were purified by centrifugation through a glycerol step gradient as determined by Sambrook and Russell (2001) after purified particles were subjected to SDS-PAGE on precast 4-15% gradient TRIS acrylamidegels (BioRad) along with protein molecular weight markers (Kropinski et al., 2012). The phage suspensions (approximately 1010 pfu/ml) were boiled for 5 min and separated by SDS-PAGE.

Denaturation, annealing and extension

The PCR and oligonucleotide primers were as described by Olsetetart et al. (2001). The consensus primers that were used to amplify the central portion of gene gp23 of the various T4-type phages were: Mzia1 (5´-TGTTATATIGGTATGGTICGICGTGCTAT-3´) and (5´-TGAAGT TACCTTCACCACGACCGG-3´). The primers that were initially used to amplify the gens gp18 analogue of the T4-type phage were: FT18-N2 (5´-GGTAAATTCCAATGGGGTCCAGCT T-3´) and (5´-TATCAGCAGCCAACGGAACC CAA-3´).

PCR sequencing

The PCR products were purified (Casjens et al., 2004) and sequenced with an Amersham Life Science Thermo Sequenase kit. The gene 18 and 23 nucleotide sequences of the various T4-type phages were determined by Mzia1 and FT18-N2 primer.

RESULTS

Bacteriophage isolation and morphology

Two bacteriophages were isolated from fecal sample and, named JH11 and JH14. All the bacteriophage produced similar plaques that were clear and medium sized 3.0-3.5 mm in diameter (Figure 1).

Electron microscopy confirmed that phages JH11 and JH14 belong to the *Myoviridae* family. Phages possessed icosahedral heads, necks and contractile tails, with tail fibers. JH11 and JH14 belong to the order *Caudovirales*. The head dimensions for JH11 and JH14 were 120 × 70 and 100 × 80 nm, and tail dimensions were 100 × 25 and 130 × 30 nm, respectively (Figure 2).

Host range analysis and stability assays

Phage JH11 and JH14 specifically lysed piglet clinical isolates of *E. coli*, whereas lysed neither bovine clinical isolates, nor other chicken. A high proportion (58.06%, n=31) of *E. coli* piglet isolates was sensitive to phage JH11 and JH14, thus confirming its broad host range (data not show). Both phages were found to be heat sensivitive (Figure 3) as more than 50% of phage particles were killed after 30 min of incubation at 60°C, and only 10% of the phage particles were still alive after 120 min of incubation. Less than 10% phage particles survived after 30 min of incubation at 70°C.

Bacteriophage resistance to acidity and alkalinity

The two bacteriophages were highly susceptible to acidity at pH 1 - 2 and susceptible in varying degrees to overnight exposure to pH 3-4. All the bacteriophages were resistant to the range of pH 5-9. Phage JH11 appeared to be a lightly more acid resistant than the JH14 (data not show).

Nucleic acid of bacteriophages

Nucleic acid of all the two bacteriophages was a DNA. All the bacteriophages' DNA samples were digested with restriction enzyme Dra I (Figures 4 and 5).

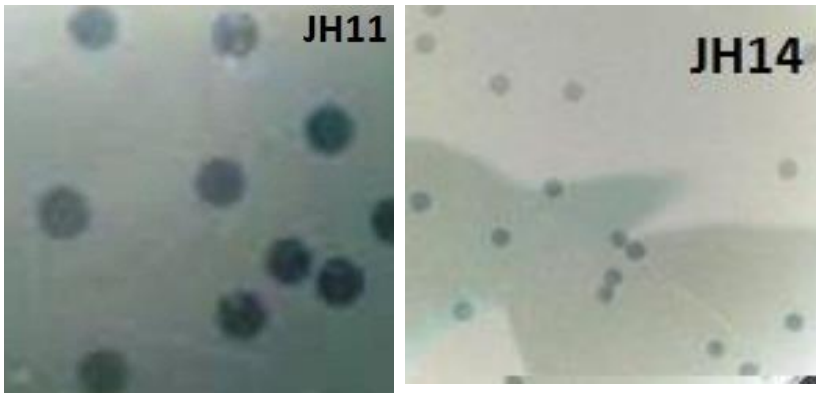

Figure 1. Plaques formed by phages JH11 and JH14 on O141 *E. coli.*

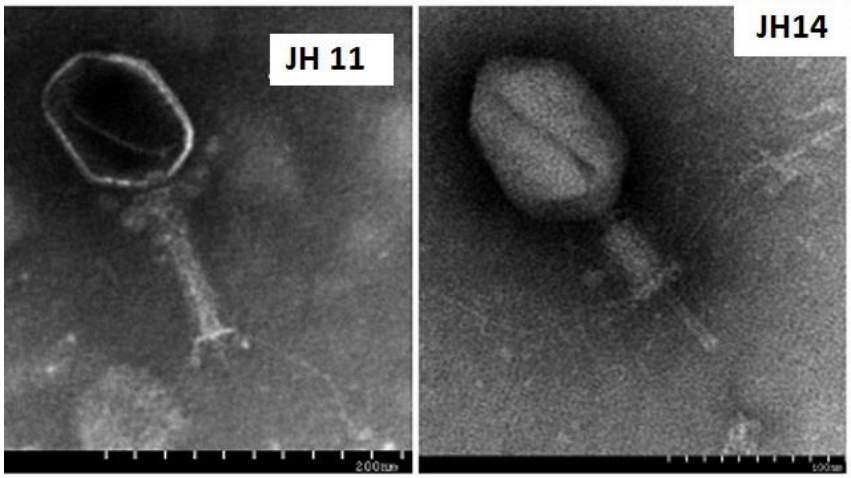

Figure 2. Appearance of phages JH11 and JH14 under electron microscopes.

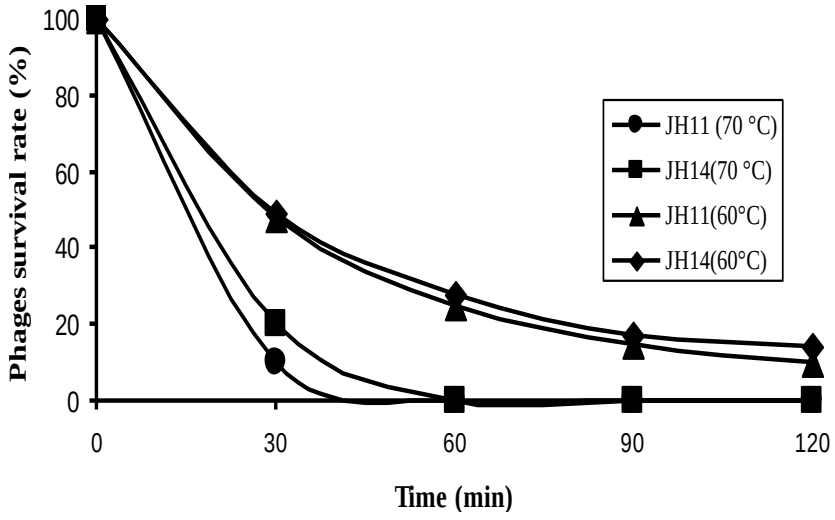

Figure 3. Thermostability of bacteriophages JH11 and JH14.

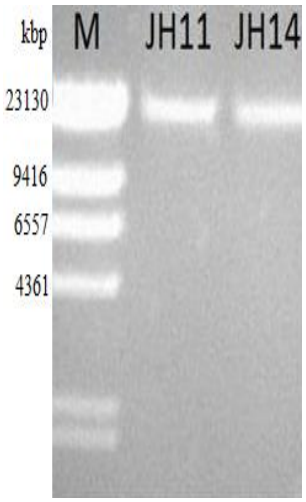

Figure 4. Electrophoresis of DNA of phages JH1 and JH14 on 0.7% agarose gels M = λ DNA marker.

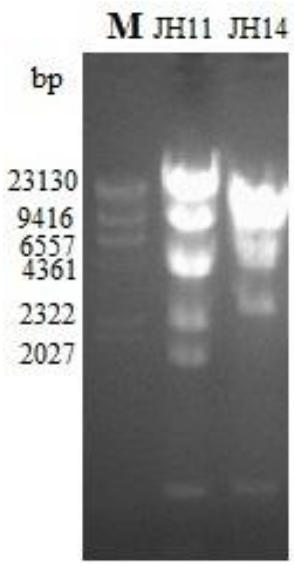

Figure 5. Analysis of phages genetic by enzyme *Dra*I on 0.7% agarose gels M = λ DNA marker.

PCR sequencing of bacteriophage JH11 and JH14 of gp18, gp23 gene

Gene gp23 on the capsid surface structure and gene gp18 were structure protein of the T4 phage. The phage JH11 was gp23 positive, also the phage JH14 gp18 was gp23 positive, this is shown in Figure 6.

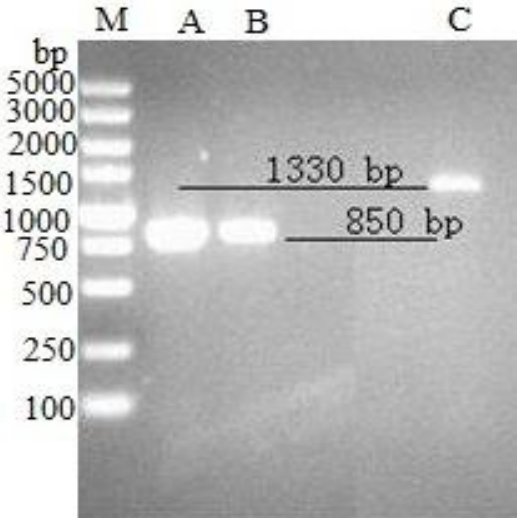

Figure 6. Agarose electrophoresis of PCR products, M: 5000 DNA marker, A: phage JH11 gene *23* PCR production, B: phage JHu14 gene gp*23* PCR production, C: phage JH14 gene gp*18* PCR production.

Blast analysis of the JH11, JH14 random clones

PCR products of gene 23 that resembled major capsid protein of JH11 and JH14 were dideoxynucleotide sequencing. Blast analysis results with the determined sequence on the NCBI website are demonstrated in Tables 1 and 2. According to the blast results, the gp23 of phage JH11 random clones were homologous with gene 23 of phage T4 MVSS (98%), ECML-134 (98%), T6 (97%) and T4 (96%) identity. Phage JH14 random clones are in close relation with HX01 (96%), CEV1 (95%) and T4 (93%). The two strains belong to T4-like phage genus. Gene of JH11 and JH14 were 93% identity (Figure 7).

Protein of purified particles of phage

Phage particles were purified and used to determine the structural protein content of each phage by SDS-PAGE analysis. Distinct profiles were observed among the two phages tested (Figure 8). Phage JH11 consisted of three abundant proteins (A, B and C) bands with approximate molecular sizes of 80, 75 and 50 kDa. Phage JH14 consisted of three abundant proteins (D, E and F) with estimated molecular size of 50, 38 and 36 kDa.

DISCUSSION

Bacteriophages are ubiquitous in our world and extremely diverse. Although recent research on bacteriophage is

Table 1. Blast analysis of bacteriophage JH11 random clones.

Accession	Random clone	Query coverage (%)	Max ident(%)
JX128259.1	Escherichia phage ECML-134, complete genome	96	98
Z78095.1	Bacteriophage T6 DNA (1506 bp)	96	97
JN202312.1	Enterobacteria phage ime09, complete genome	96	96
DQ485345.1	Enterobacteria phage MV SS major capsid protein gene, partial cds	90	98
HM137666.1	Enterobacteria phage T4T, complete genome	96	96
AF158101.6	Enterobacteria phage T4, complete genome	96	96
X01774.1	Bacteriophage T4 gene 23 for major capsid protein	96	96
K01765.1	Bacteriophage T4 genes 22 (partial) and 23 (complete cds)	96	96
DQ904452.1	Bacteriophage RB32, complete genome	96	96

Table 2. Blast analysis of bacteriophage JH14 random clones.

Accession	Random clone	Query coverage (%)	Max ident (%)
JX536493.1	Enterobacteria phage HX01, complete genome	94	96
AY331985.1	Bacteriophage CEV1 nonfunctional major capsid protein gp23	91	95
JX128259.1	Escherichia phage ECML-134, complete genome	94	93
HM137666.1	Enterobacteria phage T4T, complete genome	94	93
AF158101.6	Enterobacteria phage T4, complete genome	94	93
AY303349.1	Enterobacteria phage RB69, complete genome	94	93

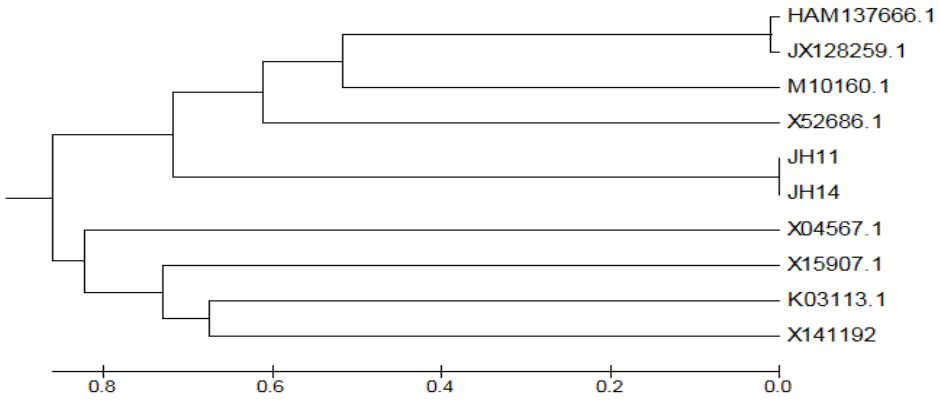

Figure 7. Dendrogram showing relationship among phage JH11, JH14, and with the determined sequence on the NCBI website.

mushrooming, it is still limited in some respects. The isolation and characterization of more bacteriophage will facilitate the utilization of bacteriophage's resources. In this study, two novel bacteriophages named JH11 and JH14 were isolated from faecal pig farm. To identify the bacteriophages taxa, PCR method was applied with the sequencing blast analysis of bacteriophage. Preliminary study with this method revealed that bacteriophage JH11 and JH14 belonged to T4-like. The goal of this study was to isolate and characterize phages against the O141 *E. coli* with special emphasis on O141: F18 ETEC, which were reported as the most common sources of coliphages from sewage samples of domestic animal drainage (pigs). Pig sewage is a collection of waste drainage for the entire pig farms and was considered to be a good source from which to isolate phages against O141: F18 ETEC,

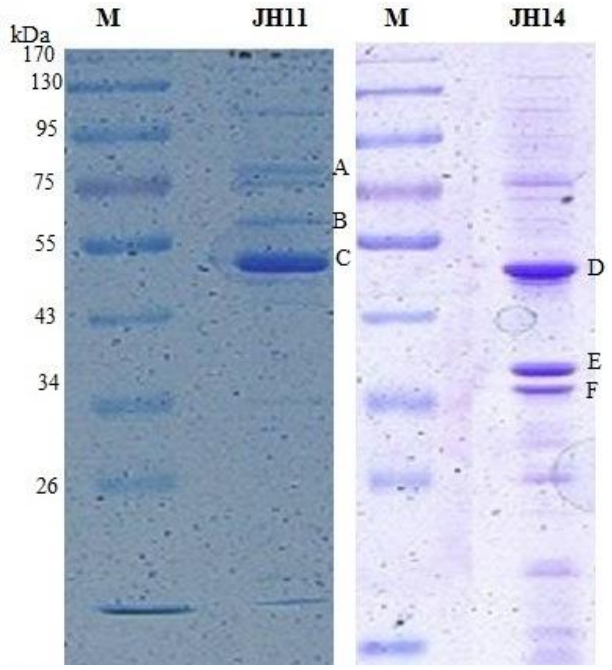

Figure 8. Structural proteins profiles of JH11 and JH14 bacteriophages by SDS-PAGE of contructural proteins of *E. coli* bacteriophages. Lanes: M: protein markers of high molecular sizes (in descending order from top to bottom, 170, 130, 95, 72, 55, 43, 34, 26, 17 and 10 kDa); 1, JH11; 2, JH12; polyacrylamide gel (12.5%wt/vol) was stained with Coomassie brilliant blue R-250.

especially when these farms had a record of infection with post-weaning *E. coli* diarrhea. To our knowledge this is the first report on isolation of phages against ETEC of any serogroup associated with PWD in pigs. Other study identified phages that were active against an O20: K101: F6 ETEC strain that causes diarrhea in neonatal pigs, and an O9: F5: ETEC that causes diarrhea in neonatal calves and lambs (Smith and Huggins, 1983). Jamalludeen et al. (2007) isolated 9 phages from raw sewage that lysed O149: H10: F4: ETEC in pig, while in chicken, 7 phages also isolated to lyse O1, O2 and O87 *E. coli* (Jamalludeen et al., 2009b). Interestingly, all the phages carried genes heat-stabile enterotoxin of the *E. coli*. Morphological characteristics were seen under an electron microscope. In the last 45 years, 96% phages of the Siphoviridae, Myoviridae, and Podoviridae family were investigated (Kumari et al., 2009). Based on morphological features and contractile tails, the phages JH11 and JH14 against O141 *E. coli* in our study were members of the Myoviridae family. This family consists of six genes, and is characterized by having icosahedral or elongated head and contractile tails that are more or less rigid, long and relatively thick (ICTV, 1995). Most of T4-like phages specifically infected certain strains of *E. coli* or some other enterobacteria. In this study, phages JH11 and JH14 genome were in order to extract as a template. 1* PCR was performed by using gene gp18 and gene gp23 specific primers in amplification. Result of agarose gel electrophoresis showed that, PCR product size was about 1330 and 850 bp, and the expected size was 2* Chao et al. (2012) concluded that T4 phage are 160 T4-like phages and more 20 protein structure genes have gene gp23 structure of the capsid surface and gene gp18 is protein structure of tail sheath. Phages were tested for their ability to lyse host ranges on the O141, O8 and O2, the predominant porcine PWD *E. coli* strains. Most of the O141 *E. coli* strains were lysed by the phage JH11 and JH14. However, were resisted by O8 ETEC and O2 ETEC. These variations might be caused by function of phage and physiological state of the host (Flayhan et al., 2012).

These two phages were highly susceptible to acidity at pH 1-2, and susceptible in varying degree to overnight exposure to pH 3-4. Phage was often quite sensitive to protein denaturation in an acidic environment, which may result in a loss of viability of the phage. The ability to survive well over the pH range between 5 and 9 was a common feature for most phages. The pH in the stomach of weaned pigs may be as low as 1-2 before a meal and may rise quickly to 4-5 after the meal, depending on the diet and the feeding regime (Snoeck et al., 2004). The two phages were likely to undergo a marked reduction in titer following oral administration to pigs unless steps were taken to reduce their exposure to low pH in the stomach and upper small intestine.

Conclusions

In this study, two phages (JH11 and JH14) that lysed O141: F1 ETEC and belong to *Myoviridae* family were successfully isolated. Boths phages were all highly active against O141: F18 ETEC. The genome sizes of these phages are larger than 20 kb. The phage JH11 was gp23 positive, while phage JH14 was gp18 and gp23 positive. The two bacteriophages belonged to T4-like bacteriophage genus. Both phages were resistant to pH 5-9, and three major protein bands were observed. These results indicated that the two phages have potential for prevention and as well as therapy for the porcine post-weaning diarrhea caused by O141: F18 ETEC.

Conflict of interest

The author(s) have not declared any conflict of interests.

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In vitro antimicrobial evaluation of two indigenous functional food-plants (*Chenopodium album* and *Solanum nigrum*) used in the Oliver Reginald (O.R.) Tambo district municipality of South Africa

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Plants have long been the source of important products with nutritional and therapeutic properties. African indigenous functional food-plants such as *Chenopodium album* and *Solanum nigrum* may constitute important sources of phytochemical constituents for the synthesis of antimicrobial compounds against infectious organisms. The objective of this study was to determine the antimicrobial properties of *C. album* and *S. nigrum* leaves used as functional food-plants in the O.R. Tambo district municipality of South Africa. Organic and aqueous solvent-extracts of *C. album* and *S. nigrum* were tested against *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (25922) and *Enterococcus faecalis* (51299) using standard microbiological techniques. Ciprofloxacin was included in all the experimental runs as positive control antibiotic. The aqueous extracts of both plants were the most active with zones of inhibition diameters ranging from 0 - 20 mm and minimum inhibitory concentration (MIC_{50}) values ranging from 0.63 - 10 mg/mL. The positive control antibiotic was highly active with zones of inhibition diameters ranging from 17 - 31 mm and MIC_{50} values from 0.0003 - 0.0005 mg/mL for all the bacteria tested. Both extracts were bactericidal with minimum bactericidal concentration (MBC) ranging from 2.5 - 20 mg/mL. From the results, it can be concluded that both plants possess compounds with antimicrobial properties, thus validating scientifically their use in traditional medicine. However, more studies to document the respective plant-principles responsible for antimicrobial activity of these plants would shed more light on their pharmacological properties.

Key words: Antimicrobial resistance, sensitivity tests, functional plants, Eastern Cape Province, South Africa.

INTRODUCTION

Bacterial antimicrobial resistance against commonly used antibiotics is distressingly on the rise (Falagas et al., 2013). Patients infected with resistant bacteria are more likely to have longer, more expensive hospital stays (Matsuura

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et al., 2013). The modification of chemotherapeutic agents to limit this problem has been greatly successful. However, many reports also indicate that many of the drugs are being rendered obsolete by microbial drug-resistance (Balsalobre et al., 2014). As a result, the treatment of microbial infection is becoming increasingly complicated. Physicians have now resorted to the use of combination therapy, increasing the cost of treatment even more. Reports on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis* infections and antibacterial resistance reveal the need for a constant search of new drugs against these organisms (Gupta et al., 2001; Tamma et al., 2012). *E. coli* is a major cause of travellers' diarrhoea, one of the most common forms of diarrhoea worldwide (Mittal et al., 2009; Canizalez-Roman et al., 2013). Both *E. coli* and *P. aeruginosa* are also major causes of urinary tract infections while *S. aureus* and *E. faecalis* are common causes of nosocomial infections (Sood et al., 2008; Oli et al., 2012). *B. subtilis* infections are not common but few cases have been reported in the literature in patients with oesophageal perforations (Jeon et al., 2012).

The use of medicinal plants in the treatment of human infections is a common practice in many remote areas of Africa with inadequate health care facilities. *Chenopodium album* and *Solanum nigrum* are functional food-plants with wide nutritional and medicinal importance among rural communities in the O.R Tambo District Municipality of South Africa (Njume et al., 2014). They are jointly referred to as *imifino ezikhulelayo* in isiXhosa, meaning indigenous vegetable. *C. album* is locally known as *imbikicane* while *S. nigrum* is known as *umsobo* (Gqaza et al., 2013a, b). Both plants grow wildly in bushes, barren land and roadside paths from where they are harvested either for nutritional or medicinal purposes. Even though they are considered as weeds in the urban areas, many rural dwellers find them tasteful and believe in their ability to alleviate problems associated with gastrointestinal discomfort. In some parts of India, *C. album* is also used in ayurveda for treating anorexia, cough, dysentery, diarrhoea, oedema, piles and worm infestations (Yadav et al., 2007). Despite their medicinal uses, very little information is available in the literature on their pharmacological potential. This is surprising considering the ever-increasing rate of antimicrobial resistance of human infectious organisms to currently used drugs. The aim of this study therefore was to investigate the antimicrobial properties of these plants in an attempt to identify cheap sources of compounds for the synthesis of new drugs against medically important bacteria.

MATERIALS AND METHODS

Bacterial strains

Standard bacterial strains including *Pseudomonas aeruginosa* ATCC 127853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 51299 and *Bacillus subtilis* ATCC 6051 (American Type Culture Collection, Rockville, MD) obtained from the stock culture of the National Health Laboratory Services (NHLS), Nelson Mandela Academic Hospital, Mthatha were used in this study. Ethical clearance was obtained from the Eastern Cape Department of Health and the Ethics Committee of the Faculty of Health Sciences, Walter Sisulu University (WSU). The bacteria were separately inoculated on Nutrient agar (Oxoid Llt., Basingstoke, UK) plates consisting of peptone (5 g/L), beef extract (3 g/L), sodium chloride (8 g/L), agar number 2 (12 g/L) at pH (7.3±0.2). The plates were incubated at 37°C for 24-48 h and examined for growth.

Collection and preparation of plant material

Mature plant-leaves of *C. album* and *S. nigrum* were harvested around the stems from home gardens and along bush paths in the vicinity of WSU main campus in Mthatha in October 2012. The plants were identified by Dr. Kathleen Immelman of the Department of Botany at WSU and voucher specimens were prepared and deposited in the Kei herbarium (CN01 and CN02). The plant-leaves were washed with tap water to remove dirt and soil particles. The plant-leaves were placed on cardboards and dried at 50°C for 24 h in a hot air oven (Heraeus, Schutzart). The plant material was powdered (ATO Mix, Cambridge) and stored in air-tight containers at 5°C for further analysis.

Preparation of plant extracts

Approximately 400 g of dried powdered plant material was exhaustively extracted in different solvents. The plant material was separately soaked in 700 mL of concentrated hexane, acetone, ethanol, methanol and water in 2 L volumetric flasks (Schott, Durban). The flasks were placed in an orbital shaker incubator (labcon, Maraisburg) for 48 h (Kuete et al., 2011). The plant material was centrifuged at 1006.2 xg for 5 min and filtered through a fritted filter funnel of pore size 60Å. The procedure was repeated twice and the three extracts combined and concentrated to dryness under vacuum (Büchi, Switzerland). The dried crude extract was collected in porcelain evaporating dish (Haldenwanger, Berlin) and left open in a biosafety class 2 cabinet (Durban, South Africa) for complete evaporation of residual solvents. The aqueous extracts were lyophilized (Castillo-Juarez et al., 2009). A 2-g sample of each extract was used for the preliminary bioassay, and where possible, another 2 g or more was put in universal bottles and kept in the extract bank. Stock solutions were prepared by dissolving the extracts in 80% acetone (a concentration we found to be non-inhibitory to any of the bacterial strains tested).

Screening of crude extracts for antibacterial activity

The agar-well diffusion method was used for this analysis (Boyanova et al., 2005). Briefly, each bacterial suspension prepared in 0.9% saline (McFarland turbidity standard 0.5) was inoculated by spreading on Mueller Hinton agar (Oxoid Llt., Basingstoke, UK) plates and allowed to dry for 15 min. Wells (6 mm in diameter) were punched into the agar using a sterile stainless steel borer and filled with 70 µL of the extract at 100 mg/mL. Seventy microliters of 0.005 mg/mL ciprofloxacin and 80% acetone were included in all experiments as positive and negative controls, respectively. The plates were incubated at 37°C for 24 h, after which the diameters of zones of inhibition were measured in mm. The experiment was repeated twice, and means for zones were recorded.

Table 1. Antimicrobial activity of crude extracts of *S. nigrum* and *C. album* as revealed by the agar-well diffusion technique.

Bacteria	*S. nigrum*					*C. album*					
	H	A	E	M	W	H	A	E	M	W	CIP
	15	0	10	0	17	0	13	0	0	14	22
Bs	11	10	10	0	20	0	21	0	0	17	29
	10	0	12	0	15	0	11	0	0	17	31
	0	0	10	0	10	0	0	0	0	11	19
Sa	7	0	10	0	12	0	0	0	0	10	19
	9	0	9	0	14	0	10	9	0	10	17
	0	0	11	0	0	0	0	0	0	17	21
Ef	0	0	15	0	0	0	0	0	0	14	19
	0	0	11	0	0	0	11	0	0	14	21
	9	0	9	0	11	0	10	9	0	9	21
Ec	0	0	10	0	10	0	0	0	0	10	18
	0	11	13	0	10	0	10	0	0	9	18
	0	0	10	0	13	0	0	0	0	15	19
Pa	0	0	13	0	12	0	0	10	0	17	27
	0	0	9	0	10	0	0	0	0	16	23
Mean± SD	4.1±5.4	1.4±3.6	10.8±1.7	0	10.3±6.0	0	5.7±6.8	1.8±3.8	0	13.3±3.1	21.6±4.2

Last row data are mean ± SD of 15 determinations for each plant crude extract; H, hexane; A, acetone; E, ethanol; M, methanol; W, water; bacteria; Bs, *Bacillus subtilis*; Sa, *Staphylococcus aureus*; Ef, *Enterococcus faecalis*; Ec, *Escherichia coli*; Pa, *Pseudomonas aeruginosa*.

Determination of minimum inhibitory concentration (50% susceptibility)

Based on their good antimicrobial activity in the screening, the aqueous extracts were selected for determination of minimum inhibitory concentration (MIC_{50}) using the micro broth dilution technique performed in 96-well plates (Bonacorsi et al., 2009). Two-fold dilutions of the extract and control antibiotic (ciprofloxacin) were prepared in the wells containing Mueller Hinton broth. The final extract concentration ranged from 20-0.31 mg/mL while that of the control antibiotic ranged from 0.005-0.00015 mg/mL. Exactly 20 µL of an 18-h old broth culture (McFarland turbidity standard 0.5) of the bacteria was inoculated into 180 µL of extract-containing culture medium. Negative control wells were prepared with culture medium only and bacteria suspension and broth only respectively. An automatic ELISA micro plate reader (Tokyo, Japan) adjusted to 590 nm was used to measure the absorbance of the plates before and after 24-h incubation. The absorbancies were compared to detect an increase or decrease in bacterial growth and the values plotted against concentration. The lowest concentration of the test extract resulting in inhibition of 50% of bacterial growth was recorded as the MIC.

Determination of minimum bactericidal concentration (MBC)

The MBC was determined following well established procedures (Nethathe and Ndip, 2011). Briefly, the entire content of the MIC well (≈200 µL) was serially ten-fold diluted in 0.9% saline. A loop-full was taken from each tube and inoculated into Mueller Hinton agar plates and incubated for 24 h at 37°C. The MBC was recorded as the lowest concentration of the extract or antibiotic that gave complete inhibition of colony formation of the test bacteria at the later cultivation.

Statistical analysis

The statistical package used for analysis was SPSS v18.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to compare the mean difference in inhibitory activities of extracts and control antibiotic, followed by Turkey's post-hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

The zones of inhibition diameters of active plant extracts ranged from 0 - 20mm while those for the control antibiotic ranged from 17-31 mm. Hexane extracts of *C. album* and methanol extracts of both plants were inactive (Table 1).

Based on agar-well results, the most active extracts (aqueous) were selected for MIC and MBC determination alongside the positive control antibiotic. The activity of the aqueous extracts was confirmed with MIC_{50} values of 0.63 - 10 and 0.63 - 7.5 mg/mL for *S. nigrum* and *C. album,* respectively (Table 2). Both extracts were also bactericidal against the tested bacteria with MBC values ranging from 2.5 and 5.0 -20 mg/mL for *C. album* and *S. nigrum,* respectively (Table 2). MIC and MBC values of 0.0003 and 0.001 mg/mL respectively were recorded for the control antibiotic and were the least values in the entire study.

DISCUSSION

There could be a double advantage in the recognition of functional food-plants as useful resources in the maintenance of good health in South Africa. Previous

Table 2. Minimum Inhibitory and bactericidal concentration (mg/mL) values of plant crude extracts and control antibiotic tested against medically important bacteria.

Bacteria species	*S. nigrum*	*C. album*	Ciprofloxacin
MIC			
B. subtilis	2.5	0.63	0.0005
S. aureus	0.63	0.63	0.0003
E. faecalis	2.5	1.3	0.0005
E. coli	5	7.5	0.0005
P. aeruginosa	10	5	0.0003
Total	4.1±3.6	3.0±3.1	0.0004±0.0001
MBC			
B. subtilis	5	2.5	0.001
S. aureus	10	2.5	0.001
E. faecalis	20	10	0.002
E. coli	10	20	0.003
P. aeruginosa	10	10	0.002
Total	11±5.5	9±7.2	0.002±0.0008

The total results shown are representative of mean ± SD value of five determinations for each plant crude extract or control antibiotic.

studies on some of these plants indicate them to be useful sources of important dietary components such as vitamins (A, B2, C, folic acid), iron, selenium, zinc, calcium and magnesium (Akubugwo et al., 2007; van der Walt et al., 2008; Gqaza et al., 2013a,b). Some of these nutrients occur in high enough quantities to meet the recommended daily allowance in children, preventing hidden hunger, its effects (stunted growth, anaemia, overweight, obesity) and promoting optimal growth and development (van Jaarsveld et al., 2014). Their medicinal properties indicate that they may also constitute an important source of antioxidants and therapeutic compounds against human infectious organisms. Many plants have been reported to contain flavonoids, alkaloids, tannins, phenols, saponins or other secondary metabolites which serve as defense mechanisms against microorganisms, insects and animals (Madduluri et al., 2013). These compounds are known to act in different ways to exert antimicrobial action. Some, such as tannins do so by binding to proline-rich proteins and interfering with protein synthesis while others such as saponins interact with bacterial cell walls to cause leakage of vital cell proteins (Shimada et al., 2006; Madduluri et al., 2013). However, this study did not evaluate the mechanism of action of the crude extracts against the tested bacteria. Nevertheless, it indicates that crude extracts of *C. album* and *S. nigrum*, especially aqueous extracts have the potential for further evaluation as antibacterial agents. The crude extracts inhibited the test bacteria, sometimes at low concentrations of 0.63 mg/mL. However, higher concentrations, at times up to 20 mg/mL were needed to completely kill the bacteria (Table 2).

Gram-positive organisms: *S. aureus*, *E. faecalis* and *B. subtilis* were the most susceptible in the entire study while Gram-negative bacteria such as *E. coli* and *P. aeruginosa* were less susceptible (Tables 1 and 2). The difference in susceptibility between Gram-negative and positive bacteria to antimicrobial agents has been reported by other researchers (Cock, 2007; Madduluri et al., 2013) and may be attributed to structural differences in the cell wall of both organisms. Gram-negative bacteria have a lipid protective sheath around their cell walls which seems to shield them from the effects of antimicrobial agents (Silhavy et al., 2010). All the bacteria tested were highly susceptible to ciprofloxacin, the control antibiotic (Tables 1 and 2). The plant crude extracts were relatively less active when compared to ciprofloxacin ($P<0.05$). This was expected as the control antibiotic is a purified compound which may also contain excipients to facilitate activity. The crude extracts on the other hand are made of numerous compounds; some of which may be synergistic or antagonistic against each other. Equally important is the fact that the quantity of the active ingredient in the crude extracts may be in minute quantities, not enough to exhibit the type of activity demonstrated by the control antibiotic. Of all the bacteria tested, *B. subtilis* and *S. aureus* were most susceptible to aqueous extracts of the plants, producing large zones of inhibition diameters (Table 1), low MIC and MBC values (Table 2). However, there were no significant differences in antibacterial activity between the aqueous extracts of *C. album* and *S. nigrum* ($P>0.05$).

Conclusion

The current study illustrates the antibacterial properties of crude extracts of *C. album* and *S. nigrum* against some selected bacteria of medical importance. The study shows that aqueous extracts of both plants are inhibitory and bactericidal to *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli* and *E. faecalis*. These findings are consistent with their folkloric use in the treatment of stomach-related morbidities in the O.R. Tambo District Municipality of South Africa. However, more studies to document the plants active ingredients will shed more light on their pharmacological relevance as antibacterial agents.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Chemical characterization and bioactivity evaluation of bacteriocin from marine biofilm-forming bacteria

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The marine biofilm bacteria were isolated on polyvinyl chloride (PVC) sheet immersed from offshore platform of Central Electrochemical Research Center (CECRI), Tuticorin, Tami Nadu. The isolated marine biofilm bacteria such as *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp., Bacillus spp., *Flavobacterium* sp., *Micrococcus* sp. and *Cytophaga* sp were identified up to generic level. The *Pseudomonas* sp. was the dominant, primary biofilm forming bacteria. The cell free crude extract of single (*Aeromonas* sp.) and co-cultured (*Aeromonas* sp. + *Enterobacter* sp.) system showed the significant repellent activity against *Pseudomonas* sp. The crude fractions were characterized by FTIR for functional group identification and GC-MS for compound identification. The results of FTIR shows different peaks like 3302.16, 2816.56, 1227.44 and 2724.02 cm^{-1} indicating the presence of bromine, amine, and phenolic, hydroxyl, amino, carbonyl and phosphoryl functional groups. It was also noted that mixed culture system produced increased level of bacteriocin as compared to single culture system. The chemical characterizations of the inhibitory molecules such as nonane, is common to both single as well as co-culture system. The higher inhibitory activity observed in co-culture system is due to the presence of nonadecane, heneicosane and cholestane compounds. The natural biogenic compounds from the marine bacteria studied have the potential to be used as a substitute to commercial biocides for anti-microfouling.

Key words: Anti-biofilm activity, bacteriocin, nonane, bioactivity, bioremediation.

INTRODUCTION

Marine structures such as ships, marine platforms, offshore rigs and jetties are under constant attack from the marine environment by fouling organisms. These structures need to be protected from the influences of the key elements of the marine environment such as saltwater, temperature fluctuations and biofouling (Chambers et al., 2006). Biofouling is one of the major significant problems and ubiquitous in the marine environment. It is a natural process of colonization of submerged surfaces, either living or artificial, involving a wide range of micro and macro organisms

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Figure 1. The Biofilm development rack.

(Briand, 2009). The attachment of microorganisms like bacteria, algae, diatoms and fungi are called microfouling (biofilm), which initiates the macrofouling. The problem of biofouling is so severe that worldwide the expenditure incurred on antifouling measures alone is approximately US$ 6.5 billion a year (Bhadury and Wright, 2004). Application of antifouling coatings like self- polishing copolymer antifouling paints (SPCs) with tributyl tin (TBT) as biocide and copper-based paints were widely employed for controlling fouling on marine structures (Chamberset al., 2006; Claisse and Alzieu, 1993; Batley et al., 1994). However, the toxicity of TBT and copper to marine organisms cause bioaccumulation of metal, imposex, etc. Nowadays, natural products from marine organisms can be used as replacements for the chemicals commonly used in antifouling coatings (Clare, 1996). Bacteriocins are antimicrobial compounds produced by bacteria that can kill closely related strains of the same species. Marine bacteria, primarily belonging to the *Pseudoalteromonas* genus, the *Vibrionaceae* family, and the *Roseobacter clade*, excrete compounds that can reduce bacterial biofilm formation and settlement by microorganisms on surfaces suggesting their suitability as antifouling bacteria (Bowman, 2007; Dobretsov and Qian, 2004). The chemotaxis activity of the bacteriocins toward the microfouling bacteria to prevent the initial biofilm formation, accordingly the prevention of biofilm would be a safe and effective way to prevent the macrofouling through eco-friendly (Armstrong et al., 2000). The main purpose of the present study was to screen the antifouling potential of marine bacterial metabolites against primary biofilm forming bacteria. Finally, chemical characterization of bacteriocin obtained from the co-culture of *Aeromonas* sp. and *Enterobacter* sp. was done.

MATERIALS AND METHODS

Marine biofilm bacterial strain

Polyvinyl chloride (PVC) sheet was cut into the dimension of 12″ ×12″ and the sheets were degreased using acetone and mounted on a wooden rack having the total size of 75″ × 15″, using brass bolt and nut. The rack was immersed at 2 m depth from the mean surface seawater below the offshore platform of Central Electro Chemical Research Institute (CECRI) at Tuticorin unit, Tamilnadu, India for 24 h (Figure 1). After 24 h, the biofilm was scrapped from PVC sheets using sterile brush and transferred to glass tube containing sterile seawater. Further the scrubbed biofilm samples was serially diluted and spread on Zobell Marine Agar and the plates were incubate up to 24 h at 37°C. The isolated biofilm bacteria were characterized using various morphological and biochemical tests (Allegrucci and Sauer, 2007; Balasubramanian et al., 2012).

Bacteriocin activity

For bacteriocin production, the isolates were inoculated by means of (i) single cell culture by *Aeromonas* sp. and co-culture by *Aeromonas*sp + *Enterobacter* sp. were prepared in Zobellmarine broth and incubated at 37°C for 24-48 h in orbital shaker at 120 rpm over night. The growth kinetics of the single as wells co-culture systems has been monitored by taking the OD_{600} starting from 0, 1, 2, 3, 4, 5, 10, 15, 20 and 24 h respectively (Sher et al**,** 2011). The bacteriocins were extracted after 48 h of culture, because the production rate is high during the exponential phase and late stationary (Hammami et al., 2009).

The cultures were centrifuged at 6000*g* and the supernatant was precipitated with ammonium sulfate at 80% saturation under chilled conditions for 18-24 h. The precipitated bacteriocins were collected by centrifugation (10,000 *g*, 30 min) and the pellet was dissolved in 50 mMTris-HCl (Lili et al., 2006). The agar well diffusion method was used for detection of bacteriocin activity in crude cell-free supernatants from single and co-culture systems against primary biofilm forming bacteria *Pseudomonas* sp. All the plates were incubated at 37°C for 24 h; the zone of inhibition was observed (Todorov and Dicks, 2005).

Analysis of natural products

The qualitative analysis by FTIR (Model RX) spectrometry has enabled us to make an identification of functional groups present in the sample of the crude extracts. The crude extracts were subjected to column chromatography over silica gel (Merck, mesh size 100-200µm) and eluted with an *n*-hexane/ethyl acetate combination (8:2). The major bioactive fractions of *n*-hexane-ethyl acetate were repeatedly run over the silica gel and these crude fractions were separated and subjected to GC-MS analysis. Analysis of natural

Table 1. The biochemical characteristics of microbial isolates.

Biochemical parameters							Suggested Genera
Gram Staining	Motility	Indole	Oxidase	CH_2O	Penicillin sensitivity	Pigmentation	
G^-	+	-	-	-	-	-	***Pseudomonas* sp.
G^-	+	+	+	AG	-	-	**Aeromonas* sp.
G^-	+	+	-	AG	-	-	**Enterobacter* sp.
G^+	+	+	-	-	-	-	**Bacillus* sp.
G^+	-	-	-	-	-	-	**Micrococcus* sp.
G^-	+	-	-	-	-	Orange	**Flavobacterium* sp.
G^-	+	-	-	-	+	Yellow	**Cytophaga* sp.

*** Dominated groups; ** Moderate; * presence; *AG* – acid and gas.

Table 2. The seawater physicochemical parameters for the study area.

Parameter	Tuticorin - Open sea
Salinity (‰)	35.00
pH	7.8
Dissolved oxygen (mg/l)	4.05
Inorganic phosphate (µmol/l)	0.725
Nitrite (µmol/)	0.0150
Nitrate (µmol/)	3.37
Ammonia (µmol/)	2.25
Calcium (mg/l)	400
Magnesium (mg/l)	1275
Total phosphorus (µmol/l)	3.27

products was carried out with a GC-MS (SHIMADZU, QP 2010) for identification of the mass proportion of active fractions (Bhattarai et al., 2007).

Crystal violet binding assay

The overnight culture of biofilm bacteria *Pseudomonas* sp. was diluted to about 1×10^6CFU/ml with fresh sterile medium. The antifouling potential of each metabolite from single and co-culture system was tested against *Pseudomonas* sp. using 96-well microtiter, the plates were gently shaken to permit growth and encourage biofilm formation. After an incubation period of 24 h, the plates were washed with PBS buffer, air dried and stained with 0.2% crystal violet solution. The plates were then again washed thoroughly to remove any unbound crystal violet dye. Ethyl alcoholwas (99.0%) then added to the wells in order to elute the crystal violet dye adhering to the biofilm (Thenmozhi et al., 2009).

RESULTS AND DISCUSSION

Seawater characteristics and bacterial identification

The isolated and identified marine bacterial isolates are *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp., *Cytophaga* sp., *Micrococcus* sp., *Bacillus* sp., *Flavobacterium* sp. Among the isolates, *Pseudomonas* sp. is found in dominant and initial biofilm forming bacteria followed by *Aeromonas* sp. and *Enterobacter* sp. (Table 1). The physico-chemical characters of the Tuticorin coastal water samples were found in normal (Table 2).

Growth kinetics of the bacteriocin producing biofilm bacteria

The growth pattern among the single and co-culture system, the mixed cultures of *Aeromonas* sp. and *Enterobacter* sp. showed increased growth rate. In both culture, bacteriocin production starts high during the exponential phase (12 h) and increased bacterial growth rate was observed in stationary phase (Boe, 1996; Bizani and Brandelli, 2002). The high bacteriocin production in exponential phase is due to increased bacterial growth rate as observed in stationary phase (Boe, 1996). It was also noted that co-cultures system shows significant increased growth rate where as single culture system shows the decrease level of growth rate. The bacteriocin from mixed culture system shows significant antagonistic activity against marine biofilm forming *Pseudomonas* sp. (Figure 2) is due to presence of more antibiofilm compounds in comparison with single cell culture system (Table 3). The prevention of the bacterial adhesion at the very initial stage, can considerably reduce the risk of further biofilm formation.

Bacteriocin activity

Bacteria from marine environment are known for their rich source of bioactive molecules, but the reports are scanty for their antibiofilm metabolites (Thenmozh et al., 2011; Bakkiyaraj and Pandian, 2010; Nithya et al., 2010). Moreover, several studies suggest many marine bacteria are capable of producing novel antibiofilm compound(s)

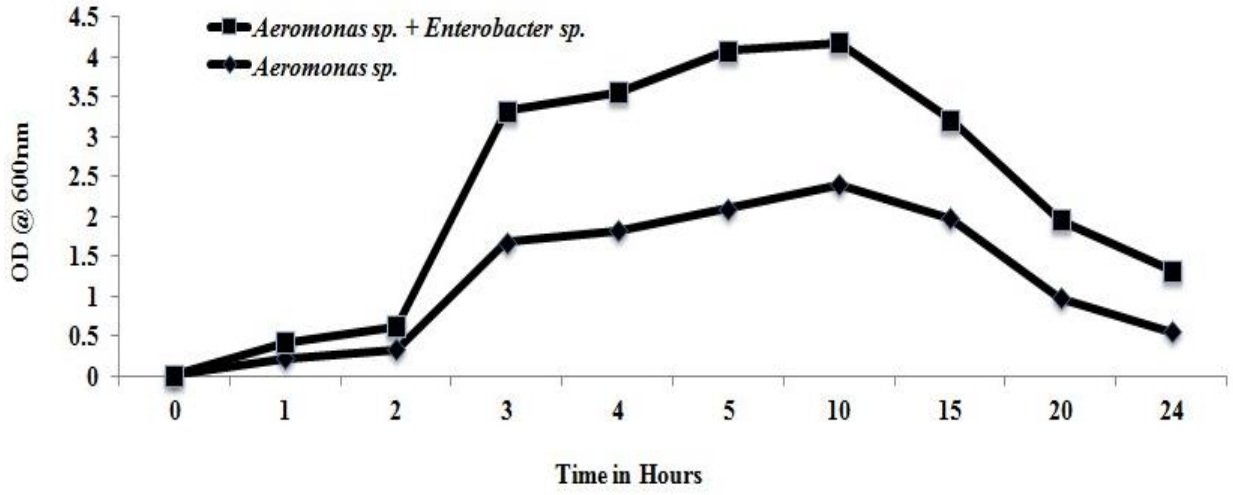

Figure 2. The growth kinetics of bacteriocin producing marine bacteria.

Table 3. FTIR spectrum of the single and co culture extracts.

S/N	Frequency (cm^{-1})	Functional group assignment	References
Single culture			
1	3302.16	H, Free NH, CH stretching vibrations,=C–H	Dumas and Miller[34]
2	2816.56	C–H of CH_3,CH_2,CH	
3	2084.11	C–C=C–C=CH stretching vibrations of $–CH_3>CH_2$	Guo[36]
4	1354.57	C □□O	Stuart[35]
Co culture			
1	3428.16	O–H AND N–H stretching vibration	Guo[36]
2	2094.80	C–C=C–C=CH	
3	2811.70	C–H stretching vibrations of $–CH_3>CH_2$	Stuart[35]
4	1601.07	C–N stretching	Guo[36]
5	1353.34	C-H of CH3, CH2 groups and ʋs C-O of COO− groups	Wolkers[37]
6	1227.44	C–N stretching	Yee *et al*[38]

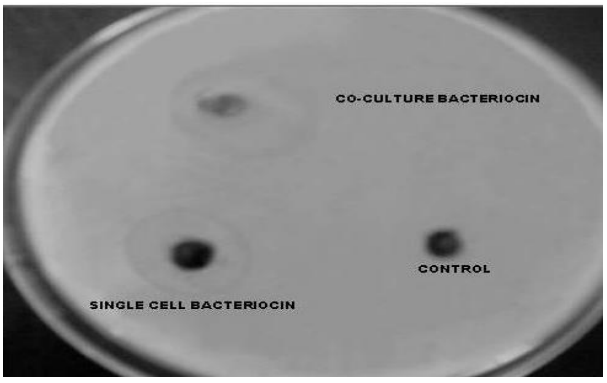

Figure 3. The bacteriocin activity against *Pseudomonas* sp.

which have not been tapped from terrestrial environment (El-Gendy et al., 2008; Selvin et al. 2009). In the present study, bacteriocin extracts obtained from marine biofilm bacteria were used to test against primary biofilm forming bacteria *Pseudomonas* sp. by using agar well method. The zone of inhibition was high in bacteriocin obtained from co-culture system as compared to single culture (Figure 3). A few anti-biofilm metabolites secreted by bacteria and devoid of antibacterial activity against free living cells were previously reported as in *E. coli* strains producing group II capsules that release a soluble polysaccharide in their environment, preventing biofilm formation by a wide range of Gram-positive and negative bacteria (Valleet al., 2006).

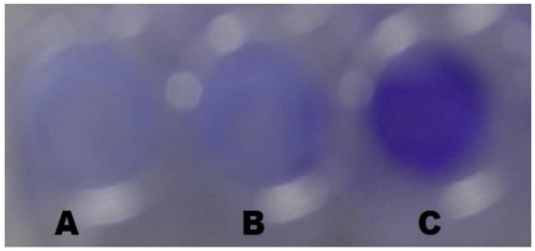

Figure 4. Biofilm stained with crystal violet.

Crystal violet binding assay

The 96-well polystyrene microtiter plate assays is a simple means of testing bacterial biofilm formation; it is a rapid and quantitative method which permits the direct quantification of adhered marine bacteria in a microtiter plate. This basic assay can be adapted readily to study several aspects of biofilm formation (Jianget al., 2011). The result of the 96-well reveals the bacteriocins from both culture systems shows significant decreased biofilm formation (Figure 4), the dark well indicates biofilm positive and light well shows biofilm negative. The well contains the culture of *Pseudomonas* sp. along with bacteriocin showing light colour when dye with crystal violet is due to the presence of anti-adherent compound in bacteriocin. The culture of *Pseudomonas* sp. alone in the well shows dark colour when dye with crystal violet indicate the biofilm formation in 96-well (Thenmozhi et al., 2011).

FTIR spectrum of the bacterial metabolites

FT-IR spectroscopy defines the chemical composition of single as well as co-culture system of the marine bacterial isolates exhibited various active metabolites (Figure 5). The functional groups spectral region (4000-400cm^{-1}) obtained from single culture systems exhibits four major peaks whereas six major peaks are observed in co-culture systems (Table3).The higher antibiofilm activity against *Pseudomonas* sp. observed in the bacteriocin of co-culture system is due to the presence of additional functional peaks like 1227.44 and 2724.02 cm^{-1} could have been active against the marine biofilm bacteria.

GC-MS analysis of the crude cell extract

The results pertaining to GC-MS analysis leads to theidentification of number of compounds from hexane and ethyl acetate extract (8:2) of single culture (*Aeromonas* sp.) and co-culture strains (*Aeromonas* sp. + *Enterobacter* sp.) clearly revealed the presence of several organic metabolites acts as a anti-biofilm in nature. Chemical characteristics of active fraction on the basis of spectral data by GC-MS were found to be a mixture of various biogenic compounds. The characterization of the inhibitory molecules exhibited in secondary metabolites such as nonane, pentadecane and hexadecane is common to both single as well as co-culture system. The compounds 3-eicosanone, cyclohexane, undecane, octadecane and docosane were exhibited in single culture system likewise iron, nonadecane, heneicosane, cholestane were exhibited in co-culture system (Table 4 and Figure 6). The higher antibiofim activity associated with co-culture's bacteriocin is due to the presence of iron, nonadecane, heneicosane and cholestane.

Sponge associated marine bacteria produced octa-decaneand their antibacterial and antilarval- settlement activity was for possible new sources of less toxic bioactive antifoulants (Dash et al., 2009). The chloroform extract of *Andrographis paniculata* exhibits the octa-decane, hexacosane, eicosane, heptadecane observed to be active against the opportunistic and pathogenic Gram negative bacteria (Roy et al., 2010). Previous report showed antimicrobial activity of nonane extracted from individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils (Pascalet al., 2002).

In the present study, the bacteriocin produced in the co-culture system shows good anti-biofilm activity against Gram negative pioneering marine biofilm forming bacteria *Pseudomonas* sp. Accordingly, the Gram negative bacterium *Pseudomonas* sp. was found to be the pioneer bacteria to colonize the marine structure. Marine bacterium *Pseudomonas rhizosphaerae* could produce potent antibacterial and antilarval secondary metabolites. The antibacterial compounds including cyclo-(Tyr-Pro), cyclo-(Tyr-Ile), cyclo-(Phe-Pro), cyclo-(Val-Pro), 3-phenyl-2-pro-penoic acid and uracil had various antibacterial activities towards five marine fouling bacteria (Qi et al., 2009). Our prime purpose was not only the identification of antibacterial activity, but also to screen the anti-biofilm activity expressed by marine bacteria producing bacteriocins. The present study suggests that marine bacterial isolates are the potential source for isolation of anti-biofilm agents as they have broad spectrum of activity against the target organisms. These metabolites can be further exploited for the use of antifouling compounds against biofouling through eco-friendly manner.

Conflict of interest

The author(s) have not declared any conflict of interests.

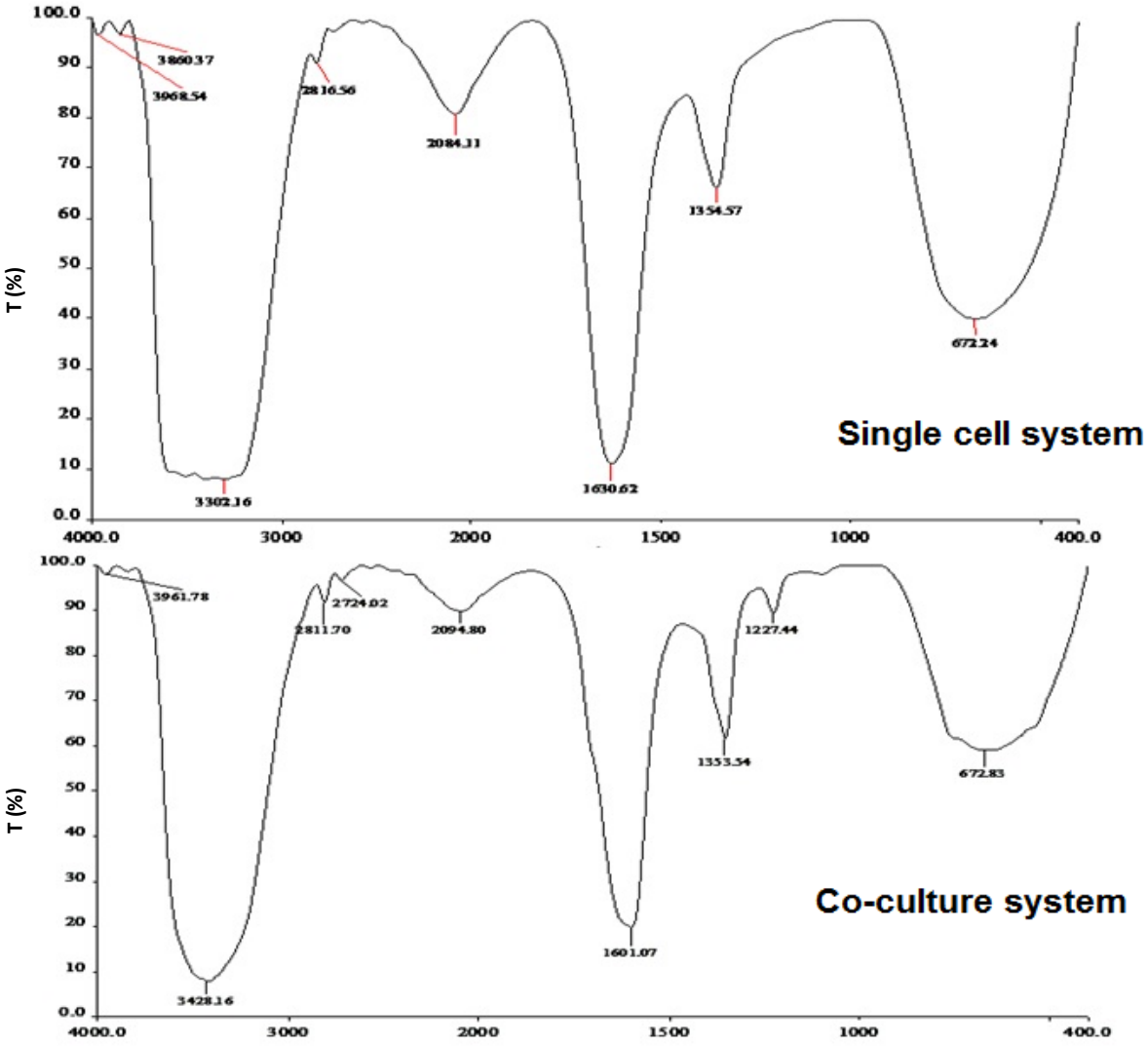

Figure 5. The FTIR spectrum of crude metabolites.

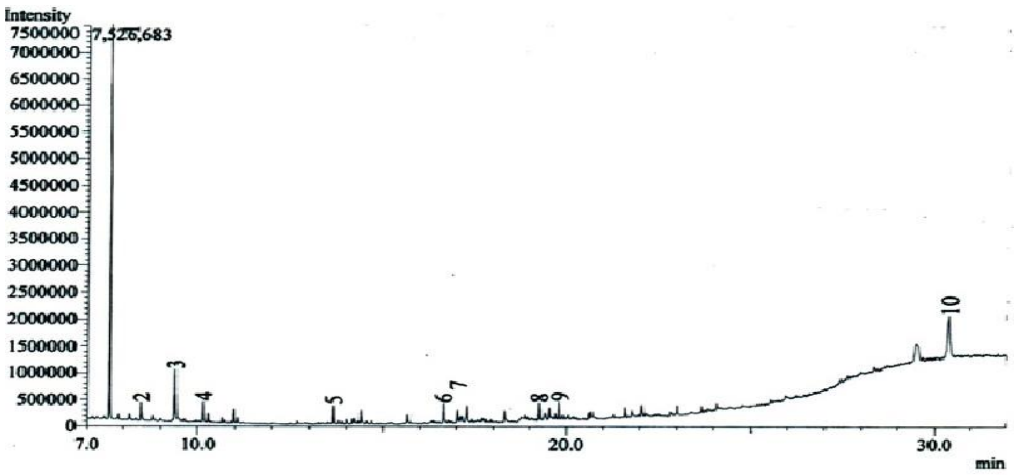

Figure 6a. The GC–MS spectrum of antibiofilm compounds in single culture systems.

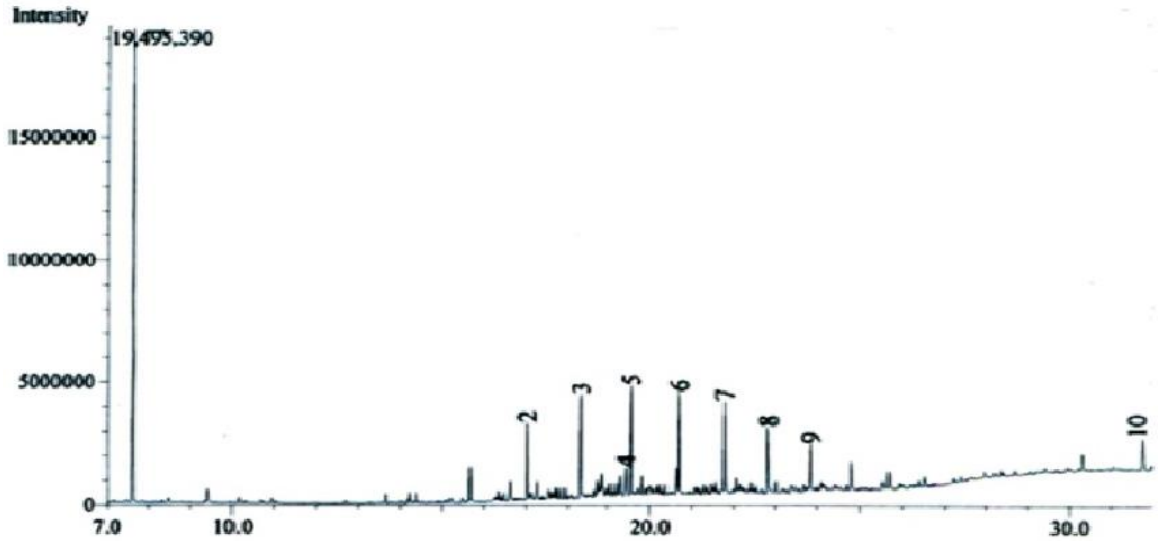

Figure 6b. The GC–MS spectrum of antibiofilm compounds in co-culture systems.

Table 4. Chemical compounds characterized by GC–MS (QP 2010 SHIMADZU).

Peak	R. Time	Area	Height	Compound	Base (m/z)
Single culture					
1	7.607	9849940	7375197	Nonane	57.05
2	8.478	599376	325846	3-Eicosanone	85.05
3	10.157	552440	368021	Undecane	57.05
4	13.651	545566	333792	Octadecane	57.00
5	16.641	515295	323444	Docosane	57.05
7	17.033	406779	216232	Pentadecane	57.10
7	19.261	490355	304833	Hexadecane	57.10
8	19.808	551578	328270	Docosane	57.00
9	30.392	3012664	684368	4-Bromo-2,6-bis(3,5-di-tert-butly-4-hydroxy)	57.05
Co-culture					
1	7.607	25707907	19331280	Nonane	57.05
2	17.027	4597973	3069066	Pentadecane	57.05
3	18.314	6196065	4086885	Hexadecane	57.05
4	19.407	2213676	1078919	Pentadecane	57.05
5	19.532	6331828	4343250	Iron	57.05
6	20.686	6321679	4070423	Nonadecane	57.05
7	21.783	5025673	3586753	Nonadecane	57.05
8	22.827	4050007	25662719	Nonadecane	57.05
9	23.824	2445399	1773641	Heneicosane	57.05
10	31.769	2561988	1202010	Cholestane	81.05

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analysis of bacteriocin samples.

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