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Full Length Research Paper

Rabies virus in biting dogs and behaviour at risk of zoonotic transmission of rabies in Ouagadougou, Burkina Faso

Dieudonné Tialla[1,2]

[1]Department Animal Health, National School of Livestock and Animal Health (ENESA), 03 BP 7026 Ouagadougou 03, Burkina Faso.
[2]Department of Biomedical and Public Health, Health Science Research Institute (IRSS), National Centre for Scientific and Technological Research (CNRST), 03 BP 7192 Ouagadougou 03, Burkina Faso.

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Rabies is an infectious, viral disease, usually inoculable by dog bite and common to humans and other mammals. It is a major zoonosis but neglected especially in Africa. However, it is the most serious and feared zoonosis in the world because once declared it leads inevitably to death. The objective of this study was to search for rabies virus in biting dogs received in 2020 at the veterinary clinic of the National School of Livestock and Animal Health and behaviour at risk of zoonotic transmission of rabies in Ouagadougou, Burkina Faso. To do this, all bite dogs conducted at the veterinary clinic of the National School of Livestock and Animal Health between January 1 and December 31, 2020 were included in the study. These dogs were observed for 15 days. The bite dogs that died during the observation were sampled. Their brains were collected aseptically and brain smears were prepared and subjected to fluorescent antibody testing. The virus was identified using the immunofluorescence technique as recommended by the World Organization for Animal Health. In total, 577 dog biters were recorded. Of the 577 biters observed, 246 42.6% [95% CI: 40.4-44.8]. Of the 246 bite dogs that died during observation 232 94.3% [95% CI: 92.1-96.5] were confirmed positive for immunofluorescence testing. Rabies virus was found in 40.2% (232/577) [95% CI: 38.2-42.2]. Test positivity was significantly associated with age, sex, breed, breeding conditions and vaccination status of the biting dog. The most common risk behaviours observed among bite dog owners were: letting children have fun with the stray and/or unvaccinated dog; letting the stray and/or unvaccinated dog lick the children's wounds; get the sores licked by the stray and/or unvaccinated dog; do not wash the sore thoroughly with soap and water after dog bite and eat the undercooked dog meat. Since rabies is a major zoonotic disease once reported, there is no treatment, adequate measures such as raising awareness among children and the general population are needed. Dog owners must vaccinate their dogs against rabies. Municipalities must be heavily involved in the fight against rabies by limiting the rambling of animals and put out of order stray dogs.

Key words: Biting dogs, Burkina Faso, Ouagadougou, public health, rabies virus, rabies, risk behaviours, zoonotic transmission.

INTRODUCTION

Rabies is a major zoonotic disease common to humans and other mammals but very neglected in Africa

(Punguyire et al., 2017). It is a highly lethal infectious disease caused by a virus that is most often inoculated by the bite of rabid dogs (Bénet and Haddad, 2004; Singh et al., 2017). Moreover, it is one of the most feared zoonoses in the World because it causes encephalomyelitis which leads inevitably to death after the appearance of symptoms (Hampson et al., 2015; Lu et al., 2018; Masiira et al., 2018). The majority of human cases worldwide are due to the bite of rabid dogs (Barrios et al., 2019; Pantha et al., 2020). It is responsible for 59,000 human deaths each year worldwide, accounting for about 44% of cases in Africa and an estimated economic loss of $8.6 billion per year (Hampson et al., 2015; Keita et al., 2020). According to the WHO, rabies is present on all continents and more than 95% of fatal human cases occur in Asia and Africa. In France, there have been 33 deaths from dog bites in the past 20 years and in most cases, the person bitten knows the dog and attacks occur at home (Lang and Klassen, 2005; De Keuster et al., 2006).

By their number and severity, dog bites are a major public health problem and only vaccination of mostly biting dogs could limit zoonotic transmission of rabies (Kaare et al., 2009; Nodari et al., 2017; Zinsstag et al., 2017). Two-thirds of the bites involve children under 15 years of age (Mège et al., 2004; Tetchi et al., 2020; Weyer et al., 2020) with two age groups, 1 to 4 and 10 to 13 years, which are most affected and most exposed to dog bites (Bordas et al., 2002; Chevallier and Sznajder, 2006; Sondo et al., 2015). They are very vulnerable to bites with lesions more specifically on the head and neck (Bordas et al., 2002; Ostanello et al., 2005), which can lead to physical, aesthetic and psychological sequelae (Kahn et al., 2003). They are also more susceptible and susceptible to dog bite-borne zoonoses because they have a less mature immune system (Afakye et al., 2016). These bites often result in thousands of emergency room calls each year (Lang and Klassen, 2005), requiring numerous hospitalizations (Bordas et al., 2002; Tan et al., 2004; Afakye et al., 2016). Thus, biting dogs pose a health hazard to populations (Adomako et al., 2018; Yizengaw et al., 2018).

In Africa, rabies has been found everywhere it has been found (Jemberu et al., 2013; Nel, 2013; Salomão et al., 2017). Thus, a synthesis of the literature with a meta-analysis of 1966-2019 data on the incidence and seroprevalence of rabies virus in humans, dogs and other animal species in 21 African countries estimated a seroprevalence of 33.8% in humans and 19.8% in animals (Wobessi et al., 2021). The prevalence was generally higher in biting dogs (Wobessi et al., 2021). In Mali, the prevalence of this condition was estimated at 90.9% in biting dogs (Traoré et al., 2020). In Kenya, 93% of bites were attributed to dogs, 78% of which were stray dogs (Ngugi et al., 2018). The disease is therefore endemic to the African continent (Reta et al., 2014; Tetchi et al., 2020). However, vaccination coverage in dogs remains very low and most dogs are stray (Hergert et al., 2016). In Burkina Faso, the dog is traditionally raised by all households either to keep the house or the herd, or to hunt, either for sacrifices at traditional funerals against the conspiracy of misfortune or for human consumption as a source of protein (Savadogo et al., 2021). But, most of the dogs are stray and go out rambling and come home only at night (Minoungou et al., 2021). Very few owners take care of their dogs with rabies vaccines (Savadogo et al., 2021; Minougou et al., 2021). However, rabies does exist in Burkina Faso with a high prevalence in stray dogs (Minougou et al., 2021). Hundreds of cases of animal bites were recorded each year and 90% of bites were attributed to dogs, of which 93.3% were stray dogs (Minougou et al., 2021). Rabies was confirmed in 78.2% of dog biters in Burkina Faso between 2008 and 2012 (Minougou et al., 2021). These dogs therefore constitute a major risk of zoonotic transmission of rabies and a permanent danger to the health of populations. The objective of this study is to investigate rabies virus in biting dogs received in 2020 at the veterinary clinic of the National School of Livestock and Animal Health and behaviour at risk of zoonotic transmission of rabies in Ouagadougou, Burkina Faso.

MATERIALS AND METHODS

Study area

The study was carried out between 1 January and 31 December 2020 at the veterinary clinic of the National School of Livestock and Animal Health of Burkina Faso. This urban clinic is located in Ouagadougou, the capital of Burkina Faso. It receives all the biting dogs of said city for a 15-day observation. It refers bitten people to the health center commonly known as the city's hygiene service for immediate management. It plays a major role in veterinary public health and is a good example of the "One Health" approach in the country.

Study population and sampling method

This study covered all bite dogs presented at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso between 1 January and 31 December 2020. The sample was systematically compiled by all the bite dogs presented at the veterinary clinic of the said school for observation during our study period. An epidemiological questionnaire was developed and completed following the information and written consent of each dog owner.

E-mail: tialladfaso@yahoo.fr.

Table 1. Individual and collective characteristics of bite dogs received at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso, 2020.

Variable		Biting dogs (n = 577)
Age group (years)	[0 - 2]	17.9% (103/577)
	> 2	82.1% (474/577)
Sex	Male	73.1% (422/577)
	Female	26.9% (155/577)
Breed	Local	96.7% (558/577)
	Exotic	3.3% (19/577)
	Stray	92.2% (532/577)
Farming conditions	Care in the home	7.8% (45/577)
	Vaccinated	15.4% (89/577)
Vaccination status	**Unvaccinated**	**84.6% (488/577)**

Diagnostic methods

Bite dogs that died during observation were sampled. Their brains were collected aseptically and brain smears were prepared and subjected to fluorescent antibody testing (FAT). The virus was identified using the immunofluorescence technique as recommended by the World Organisation for Animal Health (OIE). The most commonly used test for the diagnosis of rabies is the FAT, which is recommended by the World Health Organization (WHO) and the OIE (2013). It remains the reference technique for the diagnosis of rabies. This “baseline” test can be used directly on a smear and can also be used to confirm the presence of rabies antigens in brain tissue (OIE, 2013). FAT provides reliable results on fresh samples in a matter of hours in more than 95-99% of cases (OIE, 2013). It is also 98-100% reliable for all virus genotypes, plus it is sensitive, specific and inexpensive (OIE, 2013). For the direct diagnosis of rabies, smears were prepared from composite samples of brain tissue consisting of brain trunks and cerebellum, and attached to 100% high-quality cold acetone for at least 20 min, air-dried and then stained with a specific conjugate drop for 30 min at 37°C and stained with a cocktail of three fluorescein-labelled monoclonal antibodies to the nucleocapsid rabies virus protein (N). Slides were observed under the fluorescent microscope and positive rabies smears gave apple color fluorescence as described above (Tenzin et al., 2020; Minougou et al., 2021).

Statistical analysis

The data was entered before being imported on the software R. The variables of interest, coded in presence/absence, were positivity to the laboratory diagnostic test. The explanatory variables were individual and collective characteristics. Risk factors in dogs and risk behaviours in humans were identified using a multivariate model. A logistic regression model (proc logistic, SAS 9.3) was used to analyse positivity on the diagnostic test based on explanatory variables considered as risk factor or risk behaviour. The significance threshold was set at 5%.

Ethical consideration

This study received approval clearance from *Centre Muraz* ethical committee (number 2016-15/MS/SG/CM/IEC).

RESULTS

Individual and collective characteristics of bite dogs

As of December 31, 2020, a total of 577 bite dogs have been registered at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso. To compare young biters to older dogs, two age classes have been defined. This is Class 1 which includes animals aged 0 to 2 years and Class 2 for animals aged over 2 years. The biting dogs were divided by sex and into two breed categories: the local breed and the exotic breed. A dog was considered a stray if its owner leaves him free, he goes out and he comes home whenever he wants. The stray dogs were therefore made up of dogs left to themselves, they went out to ramble and returned home only at night. The individual and collective characteristics of the bite dogs received at the veterinary clinic of the National School of Livestock and Animal Health of Burkina Faso in 2020 are recorded in Table 1.

Prevalence of rabies in biting dogs

Of the 577 biters observed, 246 dogs died during observation, or 42.6% of biters. Of the 246 bite dogs that died during the observation 232 were confirmed positive for immunofluorescence testing or 94.3% of dead bite dogs. Rabies virus was found in 40.2% (232/577) of bite dogs received in 2020 at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso. Test positivity was significantly associated with age, sex, breed, breeding conditions and vaccination status of the biting dog.

Table 2. Prevalence of rabies by age, sex, breed, breeding conditions and vaccination status of dead biters dogs during observation at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso, 2020.

Variable	Dogs tested	Positive	Prevalence (%) and CI : 95%	p-value
Age (years)				
0-2	19	7	36.8±2.1	0.03
>2	227	225	99.1±0.9	
Total	246	232	94.3±2.4	
Sex				
Male	231	228	98.7±2.3	0.02
Female	15	4	26.6±3.2	
Total	246	232	94.3±2.4	
Breed				
Local	238	230	96.6±2.9	0.01
Exotic	8	2	25.0±3.1	
Total	246	232	94.3±2.4	
Farming conditions				
Stray	240	231	96.3±3.1	0.01
Care in the home	6	1	16.7±1.2	
Total	246	232	94.3±2.4	
Vaccination status				
Vaccinated	14	2	14.3±2.2	0.01
Unvaccinated	232	230	99.1±0.5	
Total	246	232	94.3±2.4	

CI: Confidence Interval.

Table 3. Risk factors identified in bite dogs received at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso, 2020.

Variable	OR	CI: 95%	P
Age	2.2	1.1- 4.5	0.03
Sex	1.7	1.1-2.5	0.02
Breed	1.6	1.1-2.4	0.02
Rambling dogs	1.3	1.2-1.9	0.01
Unvaccination of dogs	**1.2**	**1.3-1.7**	**0.01**

OR: Odds Ratio; CI: Confidence Interval.

Table 2 shows the different prevalence of rabies by age, the sex, breed, breeding conditions and vaccination status of dead biters dogs during observation at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso in 2020.

Identified risk factors in animals

The risk factors identified in the study animals were recorded in Table 3. Test positivity was significantly associated with age, sex, breed, dog divagation and unvaccination of dogs. These explanatory variables were considered to be identified risk factors in bite dogs.

Identified risk behaviours in humans

The most frequently observed risk behaviours among bite

Table 4. Risk Behaviours Observed in Bite Dog Owners Received at the Veterinary Clinic of the National School of Livestock and Animal Health of Ouagadougou in Burkina Faso, 2020.

Variable	OR	CI: 95%	P
Let children have fun with the stray and/or unvaccinated dog	1.8	1.2-2.8	0.04
Let the stray and/or unvaccinated dog lick the wounds of children	1.9	1.1-4.6	0.01
Getting licked wounds by stray and/or unvaccinated dogs	2.1	1.7-4.4	0.03
Do not wash the wound thoroughly with soap and water after dog bite	1.9	1.1-3.9	0.02
Eating Undercooked Dog Meat	**1.8**	**1.2-4.1**	**0.01**

OR: Odds Ratio; CI: Confidence Interval.

dog owners received in 2020 at the veterinary clinic of the National School of Livestock and Animal Health in Ouagadougou, Burkina Faso were: let the children have fun with the stray dog and/or unvaccinated; let the stray dog and/or unvaccinated lick the children's wounds; let the stray dog lick the wounds and/or unvaccinated; do not wash the wound thoroughly with soap and water after dog bite and consume undercooked dog meat. The results are presented in Table 4.

DISCUSSION

Of the bite dogs that were observed for 15 days, 42.6% of them died during their observation. This situation made us think that they were all rabid during the bites and that they would certainly have died of rage because any biting dog is suspect of rage and once declared rabies leads inevitably to death no later than 15 days after the appearance of symptoms (Hampson et al., 2015; Lu et al., 2018; Masiira et al., 2018). But after checking, rabies virus was found in 94.3% of dead dog biters. The other 5.7% would certainly have died from different diseases of rabies such as Carré's disease especially as the dogs concerned were particularly young with an age between 0 and 2 years. Considering all dog biters, the prevalence of rabies was estimated at 40.2%. This prevalence is significantly lower than that obtained (78.2%) by Minougou et al. (2021) in biting dogs in Burkina Faso between 2008 and 2012. But considering only dead biting dogs, our prevalence of 94.3% is higher than the 78.2% of Minougou et al. (2021) who considered only shot biting dogs. It is also above 90.9% obtained by Traoré et al. (2020) in Mali. These differences could therefore be explained by the fact that the dogs in our study were not only biters but they were dead during observation hence a high real prevalence of rabies while the dogs of Minougou et al. (2021) were biter dogs most often killed prior to observation. In this case, some dogs were wrongly killed while they were not suffering from rabies while they were biting. Burkina Faso is participating in the Collaborative Programme for the Elimination of Rabies by 2030 with two main measures: the vaccination campaign and passive surveillance.

Positivity on the test was significantly associated with age, sex, breed, rambling and unvaccinated of dogs and these variables were considered risk factors for biting dogs in our study. The majority of these dogs being stray dogs and most often unvaccinated the probability that they will be bitten by a rabid dog and clinically manifest rabies is very high. Moreover, contrary to what Minougou et al. (2021) found that most rabid dogs were less than four months old, in our study dogs over 2 years old and especially males were the most affected. This could be explained by the fact that during the Oestrus in females, the latter release pheromones that generally attract adult males over 2 years of age. Several males can be found around a single dog causing very fierce fights between males with bites and wounds anything that would certainly promote the transmission of rabies virus to adult male dogs over 2 years old. According to Hergert et al. (2016), in Africa the majority of dogs are stray with very low vaccination coverage, which promotes the spread of rabies and makes the continent endemic to this disease. For Ngugi et al. (2018), in Kenya 78% of bite dogs are stray dogs, often without owners, thus constituting a reservoir for the rabies virus. The local breed dogs were the most affected unlike the exotic breed dogs. In fact, in Burkina Faso, locally bred dogs have the least fortunate owners who do not take care of them. They are most often left to their own devices. They go out and ramble and come home only after dark. In addition, they are very rarely vaccinated against rabies. All this promotes contact with rabies virus and the manifestation of the disease by these dogs. On the other hand, exotic dogs are usually held by the wealthiest members of society who keep them at home. They take good care of themselves. In addition, they are vaccinated and do not leave the house to be in contact with stray and/or rabid dogs which would certainly explain the low rate of rabies virus contamination in exotic dogs. Some dogs, although vaccinated, were tested positive. This is similar to the comment made by Minougou et al. (2021). This could be explained by the specificity of the individual often leading to vaccine failures in some individuals as highlighted by Nodari et al. (2017). It is also necessary to mention the denaturation of the vaccine by heat. The protein is denatured starting at 40°C. However, in Burkina Faso,

especially during the month of April, the temperature can easily exceed 45°C in the shade. We are also witnessing repeated long-term cuts in the electric current, leading to a break in the cold chain for a long period of time. Often the vaccine is transported long distances without a cold chain. Under these conditions, the vaccine is denatured and can no longer protect. It is therefore advisable to measure the level of rabies virus antibodies sometime after each vaccination to be sure that the dog is actually protected by the vaccine.

The most frequently observed risk behaviours among bite dog owners have been to let children have fun with the stray and/or unvaccinated dog; to let the stray and/or unvaccinated dog lick children's wounds; get the sores licked by the stray and/or unvaccinated dog; do not wash the sore thoroughly with soap and water after dog bite and eat the undercooked dog meat. Indeed, some of these risk behaviours had already been highlighted by Afakye et al. (2016) in Ghana, Madjadinan et al. (2020) in Chad and Tetchi et al. (2020) in Côte d'Ivoire. The dangers associated with bites are not sufficiently known and are a real public health problem. Dog saliva can contain many pathogens including rabies virus. According to Bénet and Haddad (2004), these different pathogens can be inoculated during the bite or can be transmitted by licking a skin lesion. Therefore, getting the wounds licked by the stray and/or unvaccinated dog is a major risk of transmission of the rabies virus to humans. In Burkina Faso, the average person thinks that when you are bitten by a dog it is enough to remove the hair of the biting dog and stick it on the wound. According to them, this would allow to be protected against rabies in case the biting dog is rabid. Post-exposure prophylaxis is not controlled by the majority of bite dog owners. In addition, dog meat is widely consumed in the suburbs of Ouagadougou. These behaviours can have a negative impact on public health.

Conclusion

The objective of this study was to search for rabies virus in biting dogs received in 2020 at the veterinary clinic of the National School of Livestock and Animal Health and behaviour at risk of zoonotic transmission of rabies in Ouagadougou, Burkina Faso. Rabies virus was found in 94.3% of bitten dogs that died during observation. Positivity on the test was significantly associated with age, sex, breed, rambling and non-vaccination of dogs and these variables were considered risk factors for biting dogs in our study. On the other hand, risk behaviours have been identified among bite dog owners, including licking wounds by stray and/or unvaccinated dogs. However, the majority of bite dogs are stray and unvaccinated. Many do not know that the wound should be washed thoroughly with soap and water after a bite. In view of this situation, adequate measures such as raising children's and the general public's awareness of the danger of being in contact with a stray and unvaccinated dog and the consumption of undercooked dog meat are necessary. Vaccines must be kept in accordance with the manufacturer's recommendations without breaking the cold chain. Dog owners must vaccinate their dogs against rabies. Municipalities must be heavily involved in the fight against rabies by limiting the rambling of animals and put out of order stray dogs.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Characterization of bacterial strains from bacterial culture collection of rice sheath in Burundi highlights an *Alcaligenes* species strain with antibacterial activity against *Pseudomonas fuscovaginae* rice pathogen

Musonerimana Samson[1,3,4*], Cristina Bez[1], Habarugira Georges[2], Bigirimana Joseph[2] and Vittorio Venturi[1]

[1]International Centre for Genetic Engineering and Biotechnology, Padriciano, 99, 34149 Trieste, Italy.
[2]International Rice Research Institute (IRRI), Africa Regional Crop Improvement Office, Burundi University, Faculty of Agronomy and Bio-Engineering, Avenue de l'UNESCO N° 2 Bujumbura, Burundi.
[3]Faculty of Agronomy and Bio-Engineering, Burundi University, Avenue de l'UNESCO N° 2 Bujumbura, Burundi.
[4]Institut des Sciences Agronomiques du Burundi, Avenue de la Cathédrale 795 Bujumbura, Burundi.

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The sheath rot rice disease is known to be associated to *Pseudomonas fuscovaginae* and a recent study on the symptomatic of sheath rot revealed that this bacterial pathogen is abundant in highland rice ecology cultivation in Burundi. From asymptomatic samples of sheath rice, a collection of bacterial isolates and culturable microbiome have been carried out. A comparison between the culturable microbiome and total microbiome was made. Phenotypic assays *in vitro* on the bacterial isolates were performed after identification at the genus level of each bacterial isolate by sequencing of 16S RNA gene. A bacterial isolate belonging to *Alcaligenes* genus has antibacterial activity against the rice sheath rot pathogen *P. fuscovaginae in vitro* conditions.

Key words: *Pseudomonas fuscovaginae*, sheath rot, microbiome, *Alcaligenes* species.

INTRODUCTION

Each part of the plant (*e.g.* root, stem, leaf, and fruit) is associated with a microbial community that altogether form the plant microbiome. Depending on the plant part, the microbial community can vary considerably consisting of only a few species or being very diverse. Microbiome studies have an important role to bring insight into the composition of these communities that form the plant microbiome. The rhizospheric microbiome is the community of microbes closely associated or attached to the roots whereas the endosphere microbiome is the microbes that live inside plants in intercellular spaces mostly originating from the rhizosphere (Edwards et al.,

*Corresponding author. E-mail: smusonerimana@yahoo.fr.

2015). The phyllosphere/epiphytic microbiome is located on the surface aerial parts and lastly, the seed microbiome is the vertically transferred microbes (Gopal and Gupta, 2016).

The phyllosphere part of plants represents the largest environmental surface habitat area of microbes on earth (Lindow and Brandl, 2003; Vorholt, 2012; Peñuelas and Terradas, 2014), and much of that surface area is due to the agriculture of crops (Foley et al., 2011). Phyllosphere microorganisms or phyllosphere microbiome can be beneficial to plants by (i) increasing stress tolerance (Lindow and Leveau, 2002; Redman et al., 2002; Hamilton et al., 2012), (ii) promoting plant growth, (iii) having a role in reproduction (Doty et al., 2009; Taghavi et al., 2009; Canto and Herrera, 2012), (iv) protecting plants against aerial danger like foliar pathogens (Lee et al., 2014), and (v) can be involved in the control of flowering phenology (Wagner et al., 2014). Importantly, these microorganisms also play important roles in earth's biogeochemical cycles by moderating methanol emissions from plants (Galbally and Kirstine, 2002; Barud et al., 2016) and contributing to global nitrogen fixation (Fürnkranz et al., 2008). Despite this importance, knowledge of phyllosphere microbiomes remains relatively modest, especially for crops (Weyens et al., 2009; Vorholt, 2012; Hacquard and Schadt, 2015). To leverage, both above and below ground, plant microbiomes to support productivity and resilience of crops to environmental stresses in crops both above and below ground (Lebeis, 2014; Vandenkoornhuyse et al., 2015; Hassani et al., 2018), it is important to advance the knowledge on phyllosphere microbiome diversity and dynamics. The major roles of phyllosphere microbiome in healthy plants have been recently reviewed (Stone et al., 2018). Cultivation-independent studies have revealed that few bacterial phyla predominate in the phyllosphere of different plants and that plant factors are involved in shaping these phyllosphere communities; this is the result of specific adaptations and multipartite relationships among community members and with the host plant as reviewed by Vorholt (2012).

The rice plant (*Oryza sativa*), like other plants, has a microbial community showing differences according to the plant compartment (rhizospheric: root and phyllosphere: stem, leaves, sheath that protect the panicles). In the last decade, several studies reported an emerging rice disease that affects the phyllosphere part of the sheath tissue; the disease is called rice sheath rot. This disease has been mainly associated to *Pseudomonas fuscovaginae* which is a rice seedborne pathogen.

Microbiome and pathobiome studies on rice sheath rot have revealed that *P. fuscovaginae* is much more abundantly present in symptomatic rice plant samples with respect to asymptomatic samples (Musonerimana et al., 2020). Asymptomatic rice samples of the same rice variety in the same area/fields may possess a phyllosphere microbiome that promotes plant health and helps the plant fight sheath rot pathogen invasion. It was therefore of interest to perform an analysis of the culturable microbiome, create a bacterial culture collection isolates and characterize bacterial isolates from healthy/asymptomatic sheath rice plant samples.

MATERIALS AND METHODS

Rice samples information

The asymptomatic samples of rice used to perform the analysis of the culturable microbiome and for the isolation of bacteria in order to create a culturable collection of possible *P. fuscovaginae* antagonists were collected in two different rice growing seasons; the wet season (2017) and the dry season (2018) in Burundi. The collected plant sheath samples were not surface sterilized. 1 g of each sample was then macerated in the presence of PBS and was stored in 18% glycerol at -80°C in final volume of 1.5 ml. Before plating, 1 ml of macerated and stored at -80°C of samples were recovered and then thawed. They were transferred in PBS with final volume of 10 ml and necessary diluted at 10 times. In total, 10 asymptomatic samples were used: 6 samples from wet season and 4 from dry season. All samples were from the highland location where the pathogen *P. fuscovaginae* was reported to be predominant in symptomatic samples (Musonerimana et al., 2020).

Bacterial strains isolation

The asymptomatic samples collected and used to perform a collection of bacterial isolates were rice sheath. They were used to prepare undiluted and diluted solutions. The undiluted and the 10^{-2} diluted solution were plated on TSA (Tryptic Soy Bean: 6 g + 16 g of Agar in 1 L of sterile water) and incubated at 28°C for 2 to 3 days. The bacteria grown from the undiluted solution plates were collected en masse for the genomic DNA extraction for 16S rRNA gene amplicon community sequencing and contributed to the culturable microbiome whereas the total microbiome was from total microbial DNA isolation from the sheath rice material. Single colonies from the 10^{-2} diluted solution plates were purified by selecting those different in shape, colour and margins appearing on inoculated plates and isolates were stored at -80°C in 20% sterile glycerol.

Bacterial strains identification

The bacterial isolates were prepared for molecular identification. For this the amplification of the 16S rRNA gene was performed by using Fd1 5' AGAGTTTGATCCTGGCTCAG 3' and Rp2 5' ACGGCTACCTTGTTACGACTT 3' primers set (Weisburg et al., 1991). Colony PCR was performed after boiling (10' at 95°C) a colony suspension in 50 µL of sterile H_2O. PCR amplification was performed using GoTaq® G2 Enzyme (Promega) according to the supplier's instructions and 5 µL of template in a final volume of 50 µL was used for the PCR reaction. Reactions were performed in a T100™ Thermal Cycler (Biorad Laboratories Inc., Hercules, CA, USA) with the following thermal protocol: DNA denaturation for 5 min at 95°C, amplification (30 cycles) at 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min 30 s, extension 7 min at 72°C. Agarose gel electrophoresis was run for the PCR products and DNA from agarose was purified by using EuroGold gel extraction kit (Euroclone SpA, Italy) according to the instructions of the manufacturer. The purified PCR DNA products (16S rRNA gene) were then sequenced with the 907F AAACTCAAAGGAATTGACG

universal primer by Eurofins Genomics (Germany). Identification of the bacterial isolates at the genus level was obtained by BLAST analysis at NCBI (http://www.ncbi.nlm.nih.gov).

In vitro phenotypic assays

The bacterial isolates were tested for several *in vitro* phenotypes presumptive test. Assays for antibacterial activity against the rice bacterial plant pathogen *P. fuscovaginae* were performed by well diffusion, the culture and supernatant of each bacterial isolates were put in the middle of the TSA medium mixed with the pathogen at 0.1%; isolates were checked for lipolytic activity by streaking the bacterial isolates on 6 times diluted TSA medium amended with 1% tributyrin (Smeltzer et al., 1992); the proteolytic activity was tested by streaking the bacterial isolates on 6 times diluted TSA medium amended with 2% of powder milk (Huber et al., 2001); the exopolysaccharides (EPS) production was estimated by streaking the bacterial isolates on Yeast Extract Mannitol medium (Zlosnik et al., 2008); the indole acetic acid (IAA) production was tested by streaking the bacterial isolates on nitrocellulose membranes placed on TSA medium plates containing 5 mM tryptophan, incubating them for 24 h at 28°C and then removing the nitrocellulose membranes from TSA to place them onto a saturated Whatman paper that was previously treated with the Salkowski reagent (Bric et al., 1991); the IAA production resulted in the formation of a red/purple halo around the streak line growth of the bacterial isolates. Acyl homoserine lactones (AHLs) production was analysed by T-streak technique (Steindler and Venturi, 2007) using the biosensor *Chromobacterium violaceum* CV026 after incubation for 1 to 2 days. Motility was checked on M8 medium plates with 0.3% (swimming) or 0.5% (swarming) agar (Kohler et al., 2000).

Culturable microbiome and total microbiome analysis

Bacterial genomic DNA extraction

Bacterial genomic DNA extraction was performed from the culturable bacteria isolated from rice plant samples. The undiluted suspensions from 10 asymptomatic rice plant samples were plated on TSA medium and incubated at 28°C for 3 days. The bacteria grown were collected in 2 ml of PBS and used for genomic DNA extraction according to the Bacterial Genomic DNA extraction Kit instructions (QIAGEN, Hilden, D). The genomic DNAs extracted from 10 samples, 6 from the wet season and 4 from the dry season, were used to perform the 16S rRNA gene amplicon library as described subsequently. The same samples were used for performing the total microbiome as described by Musonerimana et al. (2020).

16S rRNA gene amplicon library preparation

The 16S rRNA gene amplicon library was performed by using the following primers: 16S Illumina library FW 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG and 16S Illumina library RW 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACH VGGGTATCTAAATCC. A mix of 2.5 µl (5 ng/µl) microbial DNA, 5 µL (1 µM) of each primers and 12.5 µl of KAPA HiFi HotStart ReadyMix in final volume of 25 µl was used for the first PCR to amplify the V3 and V4 regions of 16S rRNA gene by following this program: initial denaturation of 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final 5 min of extension at 72°C and hold at 4°C.

The PCR products were cleaned as described by Illumina protocol using AMPure XP beads, and a second PCR for adding the Illumina index was set. A mix of 5 µl (PCR products), 5 µl of each Nextera XT Index Primer (N7xx and S5xx), 25 µl of 2xKAPA HiFi HotStart ReadyMix and 10 µl PCR Grade water in final volume of 50 µl and the following program was used for the second PCR, initial denaturation of 95°C for 3 min, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final 5 min of extension at 72°C and hold at 4°C. The second cleaning was done as recommended in the protocol always using AMPure XP beads.

The second cleaning amplification products of 16S rRNA gene amplicon libraries were quantified using the Qubit Kit (Invitrogen) and the quality (integrity and presence of a unique band) was confirmed by Bioanalyzer equipment (Agilent Inc., Santa Clara, CA, USA). After quantification and normalization, all PCR products were diluted to 4 nM and aliquots of 5 µl of diluted DNA from each library were pooled together and sent to sequence by Illumina Miseq sequencing platform.

Sequence data processing

FASTQ files were analysed using DADA2 v1.4.0 (Callahan et al., 2016) adapting the methods from the DADA2 Pipeline Tutorial (1.4). R version 3.5.2 was used for all analyses. Briefly, prior to analysis in DADA2, samples were demultiplexed using the QIIME 1.9.1 split_libraries_fastq.py script. The demultiplexed files were then used as the input for DADA2. Cutadapt 1.15 was used for adapter removal and quality filtering. Later quality profiles of the reads were analysed using the DADA2 function; plot Quality Profile, to determine positions at which read quality greatly decreases. Reads were then filtered and trimmed at the identified positions (truncLen=190) using the filterAndTrim function with standard parameters (maxN=0, truncQ=2, and maxEE=2). Dereplication was performed combining all identical sequencing reads into "unique sequences" with a corresponding "abundance" equal to the number of reads of that unique sequence. DADA2's error model automatically filters out singletons, removing them before the subsequent sample inference step. Sample inference was performed using the inferred error model and chimeric sequences were removed using the removeBimeraDenovo function. The Greengenes (GG) database (McDonald et al., 2012), giving a final OTU table, was used to assign bacterial taxonomy using the assignTaxonomy function with a 97% sequence similarity.

Statistical analysis

The sequence table counts and rarefaction curves were determined on sequence count files generated by the analysis pipeline. The OTU table was rarefied according to the sample with the lowest number of reads, using the Rarefy function of the GUnifrac library. The resulting OTUs were clustered at the genus taxonomic level obtaining a final number of bacterial taxa for the two samplings. Statistical analyses were performed using the vegan package version 2.5-4 (Oksanen et al., 2019) and phyloseq package (McMurdie and Holmes, 2013) in R version 3.5.2 (Team, 2014). Relative abundances of OTUs between samples were calculated.

RESULTS

Culturable phyllosphere microbiome

It was of interest to compare the total microbiome of

asymptomatic samples from highland during the two rice growing seasons (wet season: 2017 and dry season: 2018) the culturable microbiome detected under laboratory conditions performed on the same samples. In the samples collected in the wet season of 2017, 151 taxa were detected in the total microbiome from total microbial DNA isolation and 108 were detected in the culturable microbiome. In the samples collected during the dry season of 2018, 105 taxa were detected in the total microbiome and 88 taxa were detected in the culturable microbiome. Among the 151 different taxa inferred in the total microbiome from the wet season (see above), 29% of these were found to be culturable under the conditions tested here. Similarly, among the 108 taxa identified in the total microbiome from dry season, 31% of these were found to be culturable under laboratory conditions. The number of shared and unique taxa between total and culturable microbiome is shown in the Venn diagram (Figure 1a).

The number of different taxa observed in each sample and the comparison of the alpha diversity between total and culturable microbiome is as shown in Figure 1b. The difference in the mean value of different taxa observed between the total microbiome and the culturable microbiome is not significant for the asymptomatic samples from the wet season (2017) whereas is significant for the asymptomatic samples from the dry season (2018).

During the wet and dry seasons the most frequent/abundant genera among the asymptomatic samples from the highland were *Herbaspirillum*, *Curtobacterium*, *Enterococcus*, *Methylobacterium*, *Rothia*, *Chryseobacterium, Pantoea*, *Streptococcus*, *Neisseria*, *Microbacterium* and *Sphingomonas* (Table 1).

Bacterial strains isolation and identification from asymptomatic samples

It was also of interest to purify and isolate the bacteria present in asymptomatic samples since some of these could be involved in pathogen control; in total, 150 pure bacterial colonies were purified and isolated. The 16S rRNA gene amplification and sequencing enabled the classification of 58 bacterial isolates at the genus level. The 58 bacterial isolates belonged to 21 genera; among them 16 genera were also identified in the total and/or culturable microbiome study whereas surprisingly 5 were not. This result was most likely due to their very low amounts in the samples processed for culturable microbiome study. Among the 58 isolates collected, *Microbacterium*, *Bacillus*, *Sphingomonas* and *Methylobacterium* were the most predominant (Figure 2 and Table 1).

Most of the bacterial isolates belonged to *Microbacterium*, *Sphingomonas*, *Methylobacterium* and *Bacillus* genera.

In vitro phenotypes characterization of the bacterial isolates

To obtain more information on the ability of these bacterial isolates to exert a beneficial direct or indirect effect on the plant, several plant growth-promoting (PGP) activities and phenotypes were tested. The 58 bacterial isolates were tested for the following activities and phenotypes: proteolytic activity, exopolysaccharides production (EPS), antibacterial activity against *P. fuscovaginae*, IAA production, swarming and swimming motility. In summary, 24/58 bacterial isolates displayed proteolytic activity whereas no bacterial isolates displayed lipolytic activity. In addition, 17/58 bacterial isolates displayed EPS production, 7/58 displayed swimming motility, 19/58 displayed IAA production activity, 4/58 bacterial showed swarming motility and only 1/58 isolate had anti-*P. fuscovaginae* activity (Figure 3 and Table 2) which was observed on naked eyes. This latter bacterial isolate belongs to the *Alcaligenes* genus; interestingly this activity is not due to a protein since it was resistant to the strong protease pronase hence it is most likely a compound without amino acids and peptide bonds.

DISCUSSION

This study aimed to perform an analysis of the culturable microbiome, create a bacterial culture collection isolate and characterize bacterial isolates from healthy/asymptomatic sheath rice plant samples. These asymptomatic samples were collected from rice fields in Burundi, where the rice sheath rot disease is a serious issue. The total microbiome was performed on the same asymptomatic samples as presented (Musonerimana et al., 2020) and these had a high number of genera; 151 in 2017 and 105 in 2018. In comparison the culturable microbiome presented in this work revealed 108 genera in 2017 and 88 in 2018.

The comparison, between the total microbiome and culturable microbiome displayed some differences. Some genera were present in the total microbiome and not present in the culturable: most probably some genera in the total microbiome are unculturable or could not grow under the growth conditions used here or since the plant material was frozen, could not survive freezing. Surprisingly, some isolated bacteria of the culturable microbiome belong to genera that were not detected in total microbiome analysis: most likely the growth conditions used are optimal for them and besides these genera are present in very low abundance thus the total microbiome analysis did not detect them. Many of the genera in the culturable microbiome were mostly previously reported as being part of the rice phyllosphere microbiome like *Methylobacterium*, *Sphingomonas* and *Microbacterium* (Bertani et al., 2016). These bacteria that

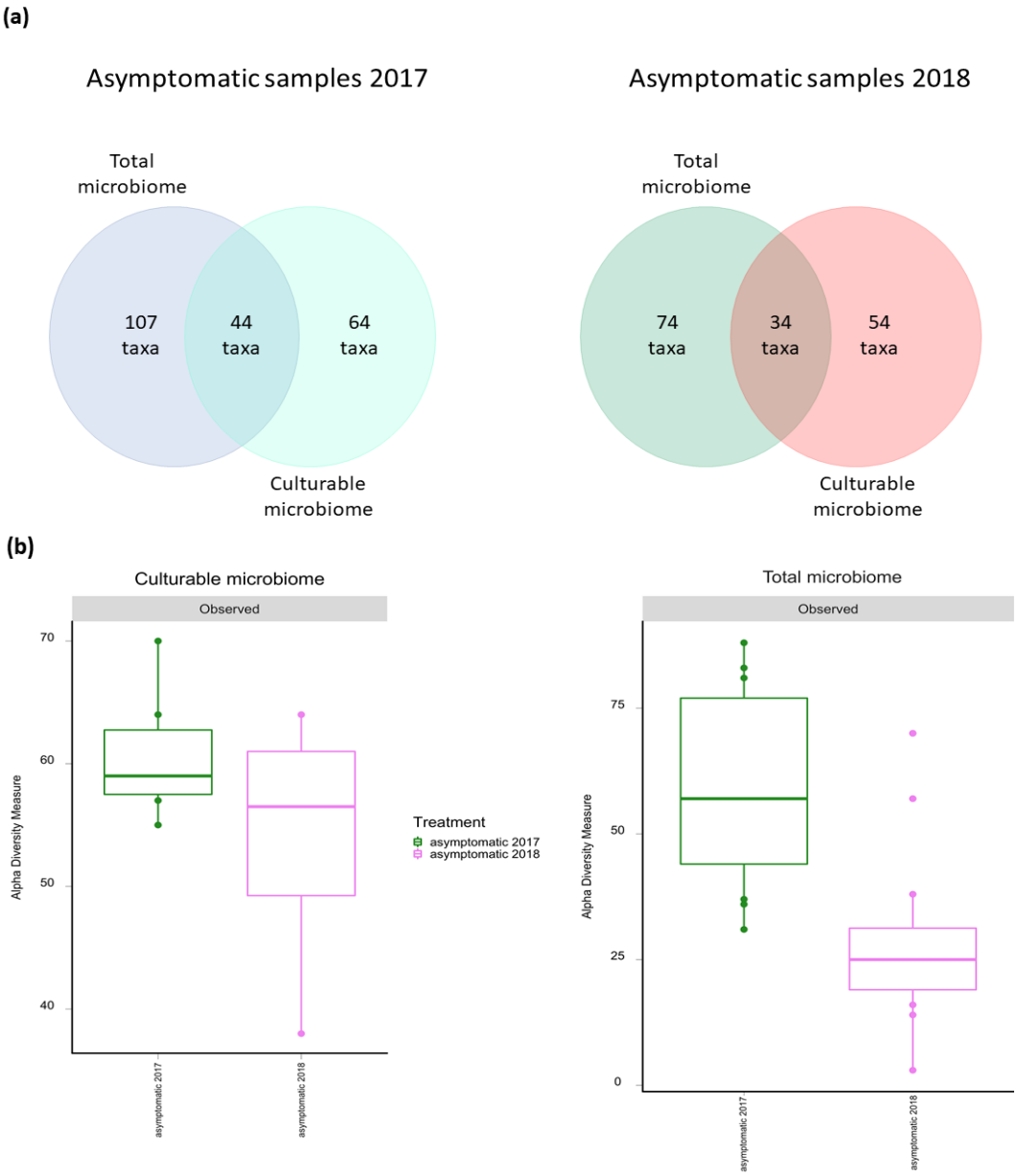

Figure 1. Total microbiome and culturable microbiome. (a) Venn diagram displaying the number of unique and shared taxa between total and culturable microbiome during the 2017 and 2018 season, respectively. (b) Alpha diversity of the total microbiome and culturable microbiome for both wet and dry seasons.

colonize the phyllosphere can adapt to a nutrient limiting environment and survive under high UV radiation (Stone et al., 2018). These members of the phyllosphere microbiome could be involved in providing the plant resistance to different stress conditions. Interestingly, the bacterial collection reported here possessed a few genera like *Alcaligenes*, *Massilia*, *Rhayibacter* that have not been reported previously to be associated with the phyllosphere of the rice plant. A possible reason is that the rice sheath samples used in this work were not surface sterilized meaning that bacterial isolates could contain endophytic and epiphytic bacteria. Many isolates belong to the genus *Microbacterium, Sphingomonas, Bacillus* and *Methylobacterium*; this could have been caused by enrichment due to the isolation conditions, especially the culture medium (TSA) and the temperature of growth.

The in *vitro* assays performed on the 58 bacterial

Table 1. Genera present in the total and culturable microbiome, bacterial isolates and identification according to 16S rRNA gene.

Genus in total microbiome	Genus culturable microbiome	Genus isolated	No. of bacterial isolates/genus
-	g__*A17*	-	-
Achromobacter	g__*Achromobacter*	-	-
Acidisoma	-	-	-
Acidovorax	g__*Acidovorax*	*Acidovorax*	1
Acinetobacter	g__*Acinetobacter*	*Acinetobacter*	3
-	g__*Actinomyces*	-	-
Actinomycetospora	-	-	-
Aeromicrobium	-	-	-
Aeromonas	-	-	-
-	g__*Agrobacterium*	-	-
-	g__*Agromyces*	-	-
-	-	*Alcaligenes*	1
-	g__*Alcanivorax*	-	-
-	g__*Alicyclobacillus*	-	-
Alkalibacterium	-	-	-
Alteromonas	-	-	-
-	g__*Ammoniphilus*	-	-
Amnibacterium	-	-	-
Anaerobacillus	-	-	-
-	g__*Anaerovorax*	-	-
Ancylobacter	-	-	-
Aquabacterium	-	-	-
Aquisphaera	-	-	-
Arcicella	-	-	-
Armatimonas	-	-	-
Arthrobacter	g__*Arthrobacter*	-	-
-	g__*Arthrospira*	-	-
-	g__*Asticcacaulis*	-	-
Aurantimonas	-	-	-
Aureimonas	-	*Aureimonas*	1
-	g__*Azohydromonas*	-	-
-	g__*Azorhizobium*	-	-
-	g__*Azospirillum*	-	-
Bacillus	g__*Bacillus*	*Bacillus*	9
Balneimonas	-	-	-
Bdellovibrio	-	-	-
Beijerinckia	-	-	-
Belnapia	-	-	-
Bosea	-	-	-
-	g__*Brevibacillus*	-	-
-	g__*Brevibacterium*	-	-
Brevundimonas	g__*Brevundimonas*	-	-
Burkholderia	-	-	-
-	g__*Caldicoprobacter*	-	-
-	g__*Candidatus Phytoplasma*	-	-
Caulobacter	g__*Caulobacter*	-	-
-	g__*Cellvibrio*	-	-

Table 1. Contd.

Chitinophaga	g__Chitinophaga	-	-
Chryseobacterium	g__*Chryseobacterium*	*Chryseobacterium*	2
Citrobacter	-	-	-
Clostridium	g__*Clostridium*	-	-
-	g__*Cohnella*	-	-
Comamonas	-	-	-
Conexibacter	g__*Conexibacter*	-	-
-	g__*Coprococcus*	-	-
Corynebacterium	g__*Corynebacterium*	-	-
Croceicoccus	-	-	-
-	g__*Cupriavidus*	-	-
Curtobacterium	g__*Curtobacterium*	*Curtobacterium*	2
Curvibacter	-	-	-
Deinococcus	g__*Deinococcus*	*Deinococcus*	1
Delftia	-	-	-
Devosia	g__*Devosia*	-	
Diaphorobacter	g__*Diaphorobacter*	-	-
Dickeya	-	-	-
Duganella	-	-	-
Dyadobacter	g__*Dyadobacter*	-	-
Elizabethkingia	-	-	-
-	g__*Emticicia*	-	-
Enhydrobacter	-	-	-
Ensifer	-	-	-
Enterobacter	g__*Enterobacter*	-	-
Enterococcus	g__*Enterococcus*	-	-
Erwinia	-	-	-
Escherichia/Shigella	-	-	-
Ethanoligenens	-	-	-
Exiguobacterium	-	-	-
Extensimonas	-	-	-
Falsibacillus	-	-	-
Ferruginibacter	-	-	-
Fibrella	-	-	-
Fimbriimonas	g__*Fimbriimonas*	-	-
-	g__*Flavihumibacter*	-	-
Flavobacterium	g__*Flavobacterium*	-	-
-	g__*Fluviicola*	-	-
Friedmanniella	-	-	-
Fructobacillus	-	-	-
Gardnerella	-	-	-
Gemella	-	-	-
-	g__*Gemmata*	-	-
Geodermatophilus	-	-	-
-	g__*Gemmatimonas*	-	-
Gibbsiella	-	-	-
-	g__*Glycomyces*	-	-
-	g__*Gracilibacter*	-	-
Haemophilus	-	-	-
Halomonas	-	-	-

Table 1. Contd.

Hartmannibacter	-	-	-
Hephaestia	-	-	-
Herbaspirillum	g__*Herbaspirillum*	-	-
Herbiconiux	-	-	-
-	-	*Huakuichenia*	1
-	-	*Humibacter*	1
-	g__*Hydrogenophaga*	-	-
Hymenobacter	-	-	-
	g__*Hyphomicrobium*	-	-
Janthinobacterium	-	-	-
Jatrophihabitans	-	-	-
-	g__*Kaistobacter*	-	-
-	g__*Kibdelosporangium*	-	-
Kineococcus	-	-	-
Klebsiella	-	-	-
Kocuria	g__*Kocuria*	-	-
Kosakonia	-	-	-
-	g__*Kribbella*	-	-
Labilithrix	-	-	-
Lactobacillus	g__*Lactobacillus*	-	-
Larkinella	g__*Larkinella*	-	-
-	g__*Lautropia*	-	-
-	g__*Leadbetterella*	-	-
Leclercia	-	-	-
Legionella	-	-	-
Leifsonia	-	-	-
-	g__*Lentzea*	-	-
Leucobacter	-	-	-
	g__*Luteimonas*	-	-
Luteolibacter	g__*Luteolibacter*	-	-
Lysinibacillus	-	-	-
-	g__*Lysobacter*	-	-
-	g__*Magnetospirillum*	-	-
Massilia	g__*Massilia*	*Massilia*	1
Mesorhizobium	g__*Mesorhizobium*	-	-
-	g__*Methylibium*	-	-
Methylobacterium	g__*Methylobacterium*	*Methylobacterium*	5
Methylophilus	-	-	-
-	g__*Methylotenera*	-	-
-	g__*Methyloversatilis*	-	-
Microbacterium	g__*Microbacterium*	*Microbacterium*	12
Micrococcus	g__*Micrococcus*	-	-
Microvirgula	-	-	-
Mitsuaria	-	-	-
Mucilaginibacter	-	-	-
Mumia	-	-	-
Mycetocola	-	-	-
Mycobacterium	-	-	-
Mycoplana	g__*Mycoplana*	-	-
-	-	*Naasia*	1

Table 1. Contd.

Nakamurella	-	-	-
Naxibacter	-	-	-
-	g__*Neisseria*	-	-
Neochlamydia	-	-	-
Neorhizobium	-	-	-
-	g__*Niabella*	-	-
-	g__*Niastella*	-	-
Nocardioides	g__*Nocardioides*	-	-
-	g__*Nocardiopsis*	-	-
-	*g__Nonomuraea*	-	-
Novosphingobium	g__*Novosphingobium*	*Novosphingobium*	1
Nubsella	-	-	-
Oceanobacillus	-	-	-
Ochrobactrum	-	-	-
Okibacterium	-	-	-
-	g__*Opitutus*	-	-
Orientia	-	-	-
Paenibacillus	g__*Paenibacillus*	-	-
Pantoea	g__*Pantoea*	*Pantoea*	1
Parachlamydia	-	-	-
Parachlamydia	-	-	-
Paracoccus	g__*Paracoccus*	-	-
Patulibacter	-	-	-
Pedobacter	g__*Pedobacter*	-	-
Pelomonas	-	-	-
Peptoniphilus	-	-	-
Peredibacter	-	-	-
-	g__*Phaeospirillum*	-	-
-	g__*Phenylobacterium*	-	-
-	g__*Phyllobacterium*	-	-
-	g__*Pirellula*	-	-
-	g__*Planctomyces*	-	-
-	g__*planctomycete*	-	-
Pluralibacter	-	-	-
Polaromonas	-	-	-
Propionibacterium	g__*Propionibacterium*	-	-
Prosthecobacter	g__*Prosthecobacter*	-	-
Providencia	-	-	-
Pseudacidovorax	g__*Pseudacidovorax*	-	-
Pseudochrobactrum	-	-	-
Pseudomonas	g__*Pseudomonas*	-	-
Pseudophaeobacter	-	-	-
-	g__*Pseudonocardia*	-	-
-	g__*Pseudonocardia*	-	-
-	g__*Pseudoxanthomonas*	-	-
Quadrisphaera	-	-	-
Ralstonia	-	-	-
Rathayibacter	-	*Rathayibacter*	1
Rhizobacter	-	-	-
Rhizobium	g__*Rhizobium*	-	-

Table 1. Contd.

Rhizorhabdus	-	-	-
-	g__*Rhodobacter*	-	-
Rhodanobacter	-	-	-
Rhodococcus	g__*Rhodococcus*	-	-
-	g__*Rhodoplanes*	-	-
Rhodopseudomonas	-	-	-
Rickettsia	-	-	-
Rivibacter	-	-	-
Roseateles	-	-	-
Roseomonas	g__*Roseomonas*	-	-
-	g__*Rothia*	-	-
-	g__*Rubrivivax*	-	-
Rudanella	-	-	-
Salirhabdus	-	-	-
Salmonella	-	-	-
Samsonia	-	-	-
-	g__*Sedimentibacter*	-	-
Segniliparus	-	-	-
Serpens	g__*Serpens*	-	-
Serratia	-	-	-
-	-	*Siccibacter*	1
Shimwellia	-	-	-
-	g__*Shinella*	-	-
Simonsiella	-	-	-
Siphonobacter	-	-	-
-	g__*Solimonas*	-	-
Snodgrassella	-	-	-
Soonwooa	-	-	-
Sphingobacterium	g__*Sphingobacterium*	-	-
Sphingobium	g__*Sphingobium*	-	-
Sphingomonas	g__*Sphingomonas*	*Sphingomonas*	9
Sphingopyxis	-	-	-
Spirosoma	g__*Spirosoma*	-	-
Staphylococcus	g__*Staphylococcus*	-	-
Stenotrophomonas	g__*Stenotrophomonas*	-	-
-	g__*Steroidobacter*	-	-
Streptococcus	g__*Streptococcus*	*Streptococcus*	3
-	g__*Streptomyces*	-	-
-	g__*Symbiobacterium*	-	-
Taibaiella	-	-	-
Tepidisphaera	-	-	-
Terrabacter	-	-	-
-	g__*Terrimonas*	-	-
-	g__*Thermomonas*	-	-
Variovorax	g__*Variovorax*	*Variovorax*	1
-	g__*Verrucomicrobium*	-	-
Williamsia	-	-	-
Xanthomonas	g__*Xanthomonas*	-	-
Yokenella	-	-	-

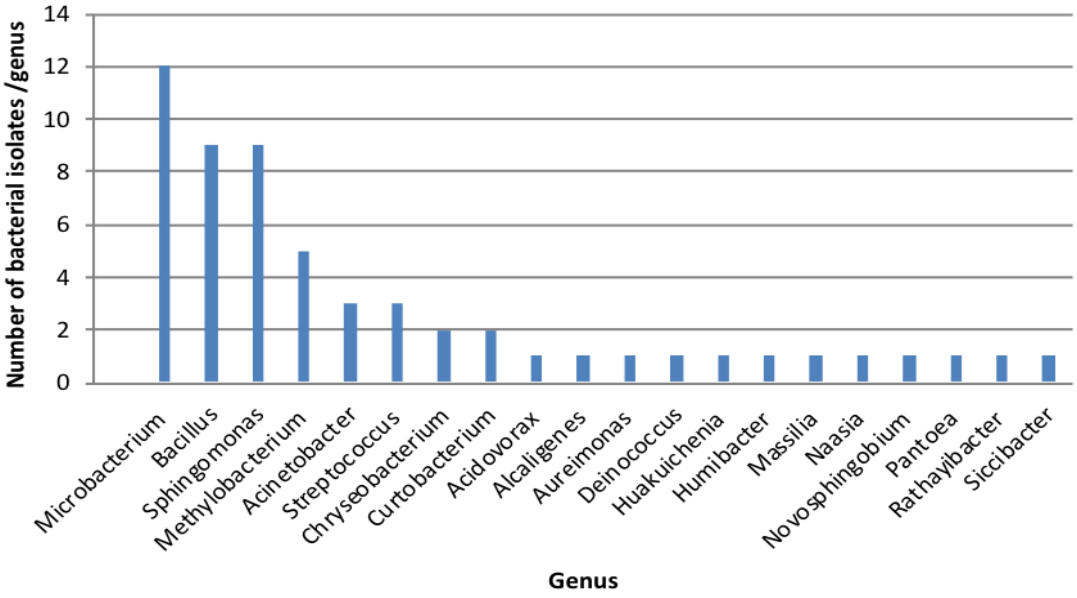

Figure 2. Diagram of bacterial isolates from asymptomatic samples; they are represented at genus level.

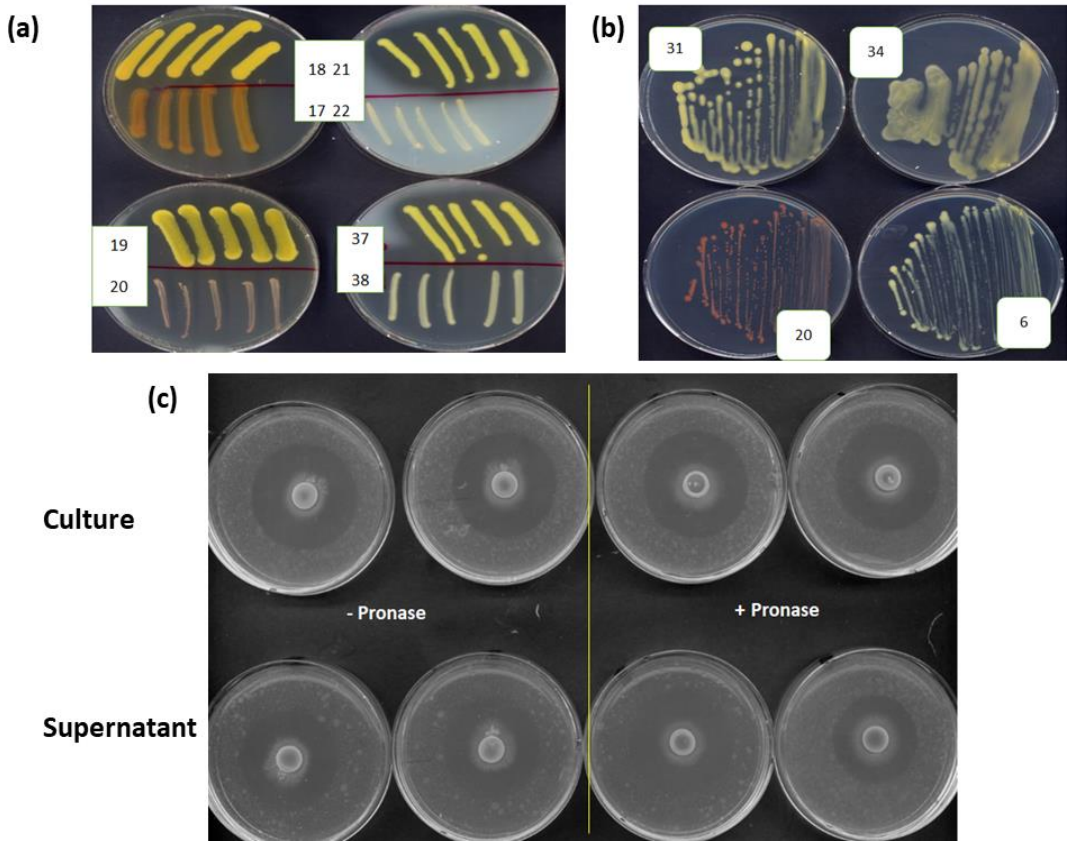

Figure 3. Phenotypes assay of the bacterial isolates. (a) Proteolytic activity assay; (b) Exopolysaccharides production assay; (c) *Alcaligenes* spp. antibacterial activity against *P. fuscovaginae* (*P. fuscovaginae* is mixed in the medium and the *Alcaligenes* spp. or its supernatant were in the center of the plate) in the presence or not of pronase. Both the *Alcaligenes* bacterial culture alive and its supernatant were used.

Table 2. In *vitro* assays on the bacterial isolates from rice asymptomatic samples of sheath rot disease.

Number	Bacterial isolates	Proteolytic activity	EPS	Antimicrobial activity	Swimming	Swarming	IAA
1	*Acidovorax* spp.	-	+++	-	-	-	-
2	*Acinetobacter* spp.	+	-	-	++	+	-
3	*Acinetobacter* spp.	+	-	-	++	+	+
4	*Acinetobacter* spp.	-	+++	-	-	-	+
5	*Alcaligenes* spp.	-	-	+	++	+	++
6	*Aureimonas* spp.	-	+	-	-	-	+
7	*Bacillus* spp.	-	-	-	-	-	-
8	*Bacillus* spp.	-	-	-	-	-	++
9	*Bacillus* spp.	++	-	-	-	-	+
10	*Bacillus* spp.	++	-	-	-	-	+
11	*Bacillus* spp.	-	-	-	-	-	+
12	*Bacillus* spp.	-	-	-	+	-	++
13	*Bacillus* spp.	-	-	-	+	-	-
14	*Bacillus* spp.	++	-	-	-	-	-
15	*Bacillus* spp.	++	-	-	-	-	+
16	*Chryseobacterium* spp.	+++	-	-	-	-	-
17	*Chryseobacterium* spp.	+++	-	-	-	-	-
18	*Curtobacterium* spp.	+++	-	-	-	-	-
19	*Curtobacterium* spp.	+++	-	-	-	-	-
20	*Deinococcus* spp.	+++	+	-	-	-	-
21	*Huakuichenia* spp.	++	-	-	-	-	-
22	*Humibacter* spp.	-	-	-	-	-	++
23	*Massilia* spp.	++	+++	-	-	-	-
24	*Methylobacterium* spp.	-	-	-	-	-	-
25	*Methylobacterium* spp.	-	-	-	-	-	-
26	*Methylobacterium* spp.	-	-	-	-	-	-
27	*Methylobacterium* spp.	-	-	-	-	-	-
28	*Methylobacterium* spp.	-	-	-	-	-	-
29	*Microbacterium* spp.	+	+	-	-	-	-
30	*Microbacterium* spp.	++	++	-	-	-	-
31	*Microbacterium* spp.	++	++	-	-	-	-
32	*Microbacterium* spp.		++	-	-	-	-
33	*Microbacterium* spp.	++	+	-	-	-	+
34	*Microbacterium* spp.	++	+	-	-	-	-
35	*Microbacterium* spp.	++	+++	-	-	-	-
36	*Microbacterium* spp.	++	+	-	-	-	-
37	*Microbacterium* spp.	+	+	-	-	-	-
38	*Microbacterium* spp.	++	++	-	-	-	-
39	*Microbacterium* spp.	+++	++	-	-	-	-
40	*Microbacterium* spp.	+++	++	-	-	-	-
41	*Naasia* spp.	-	-	-	-	-	-
42	*Novosphingobium* spp.	-	-	-	-	-	-
43	*Pantoea* spp.	-	-	-	-	-	-
44	*Rathayibacter* spp.	-	-	-	-	-	-
45	*Siccibacter* spp.	-	-	-	+++	+	++
46	*Sphingomonas* spp.	-	-	-	-	-	-
47	*Sphingomonas* spp.	+	-	-	-	-	
48	*Sphingomonas* spp.	-	-	-	-	-	++

Table 2. Contd.

49	*Sphingomonas* spp.	-	-	-	-	-	
50	*Sphingomonas* spp.	-	-	-	-	-	
51	*Sphingomonas* spp.	-	-	-	-	-	+++
52	*Sphingomonas* spp.	-	-	-	-	-	+++
53	*Sphingomonas* spp.	-	-	-	-	-	+++
54	*Sphingomonas* spp.	-	-	-	-	-	++
55	*Streptococcus* spp.	-	-	-	-	-	-
56	*Streptococcus* spp.	-	-	-	-	-	-
57	*Streptococcus* spp.	-	-	-	-	-	-
58	*Variovorax* spp.	-	-	-	+++	-	++

-: no activity, +: low activity or presence of the activity, ++: medium activity and +++: high activity.

isolates showed a diversity of phenotypes; 24 isolates had a proteolytic activity which is an important property involved in the virulence of plant pathogens (Figaj et al., 2019) as well as in the biological control of plant disease (Mota et al., 2017). 17 isolates were able to produce EPS, these molecules are known to be produced also by some plant pathogens like *Pseudomonas* and the EPS produced by *Pseudomonas syringae* is involved in biofilm formation, virulence and epiphytic fitness (Yu et al., 1999; Laue et al., 2006). It is possible that bacterial EPS provides some protection to the plant, both from desiccation and from UV damage. Biofilms in the phyllosphere may provide resistance to desiccation unlike those found in water; for example, *Pseudomonas putida* biofilms grown in air retained their morphology better after drying than biofilms were grown in liquid medium (Auerbach et al., 2000). *Pseudomonas* species are often dominant constituents of the phyllosphere suggesting that naturally occurring biofilms may limit the loss of water and exposure to UV radiation. Plants are exposed to high levels of UV radiation and can suffer developmental and genetic damage (Jansen et al., 1998). Pigmented bacteria are more UV resistant, and the phyllosphere microbiome as a whole becomes more UV tolerant towards the end of the growing season (Jacobs and Sundin, 2001). Phyllosphere microorganisms may provide some UV protection to the plant through pigmented compounds; interestingly several isolates (many *Microbacterium* species) produced EPS that were yellow pigmented. It is also known that EPS production is involved in the endophytic colonization of *Gluconacetobacter diazotrophicus* since EPS mutants were defective in the colonization of the rice root endosphere (Meneses et al., 2011).

IAA was produced mostly by isolates that belonged to *Bacillus* and *Sphingomonas*. The plant hormone (IAA) from phyllosphere microorganisms influences plant growth and the evidence suggests that phyllosphere microorganisms producing it could be involved in increasing plant productivity (Glick, 1995; Romero et al., 2016) and also be involved in the activity of stomata (Tanaka et al., 2006). Swimming and swarming movement was detected on a few isolates; these phenotypes can have an important role in the motility for the acquisition of nutrients.

The antimicrobial activity assay against *P. fuscovaginae* showed that only one isolate belonging to the *Alcaligenes* genus displayed a positive test *in vitro*. *Alcaligenes* species strains exist in soil, water, and environment, as well as in association with humans. The bacteria of this genus are usually non-pathogenic but occasionally can cause opportunistic human infections. Bacterial species belonging to the genus *Alcaligenes* have also demonstrated versatile pollutant bioremediation capability, including phenols (Rehfuss and Urban, 2005; Kumar et al., 2013), phenanthrene (Singleton et al., 2009) as well as having algicidal activity (Sun et al., 2015). The *in vitro* antimicrobial assay performed here excludes the isolates that attenuate/block *P. fuscovaginae* pathogenesis/ invasion via other mechanisms like competition for nutrients or quorum quenching. It is therefore likely other bacteria that live in the phyllosphere are involved in promoting plant health by keeping away pathogens like *P. fuscovaginae.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Biosafety, bacteriological quality and strategy of biopreservative administration for controlling spoilage bacteria in Thai traditional dried seafood products

Subuntith Nimrat[1]*, Pornpimon Soodsawaeng[2], Nanticha Rattanamangkalanon[3], Traimat Boonthai[2] and Verapong Vuthiphandchai[4]

[1]Department of Microbiology and Environmental Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand.
[2]Biological Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand.
[3]Applied Microbiology, Department of Microbiology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand.
[4]Department of Aquatic Science, Faculty of Science, Burapha University, Chon Buri 20131, Thailand.

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Biosafety has currently become an important public health issue in seafood industry of Thailand. In order to enhance quality of seafood products, three phases of study were established. In the first phase, biosafety of traditional processed squid sold in Chon Buri province, Thailand compiled from literatures between 2002 and 2019 was evaluated. The squid products represented a health hazard due to 86.5% samples, having total viable count (TVC) over the allowable limits imposed by Thailand and international food administration agencies. In the second phase, bacterial contamination during multiple steps related to dried seasoned squid preparation and storage was determined. TVC were low at approximately 10^2 CFU/g during a multi-step of preparation, and increased significantly ($P < 0.05$) to 10^3 CFU/g in post-prepared products during 25-day storage. Lastly, a novel preservation strategy was developed. A combination of partially purified solution from *Bacillus velezensis* BUU004 with nisin and the mixed herb extracts from hot pepper and lemongrass exhibited the strongest antibacterial efficacy observed by a significantly ($P < 0.05$) low TVC in the post-prepared squid within allowable limits recommended by the food administration authorities. This study suggests that the novel mixture has a preservative potential to improve biosafety of dried seafood products.

Key words: Squid, bacteriocin, nisin, herb extract, *Bacillus velezensis*, synergy, biosafety.

INTRODUCTION

Thailand is one of the major countries in production, consumption and trade of seafood products. A divergence of traditional seafood-based product with varying unique flavors and textures is produced in response to an increased demand of domestic consumption. Annual per capita consumption of seafood in Thailand is approximately 33 kg in 2016 (Pisuthipan, 2019). Biosafety has currently become a significant public health issue in

*Corresponding author. E-mail: subunti@buu.ac.th, subunttbt@gmail.com. Tel. +66-38-103-120

seafood industry of Thailand (Thungkao and Muangharm, 2008; Nimrat et al., 2019). In spite of an advanced technology in food preservation being applied to extend the shelf life, seafood products have been threatened by pathogens and pose a serious risk of food-borne diseases like diarrhea, gastritis, and food poisoning illnesses (Butkhot et al., 2019a). The most frequently isolated food-borne pathogens in dried seafood products are *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* (Thungkao and Muangharm, 2008; Butkhot et al., 2019a; Nimrat et al., 2019). This encourages food producers, food-safety inspectors and researchers to develop an effective biopreservative-based technology for improving the biosafety of food products. Among different approaches, supplementation of natural antimicrobial compounds, such as bacteriocins, bacteriocin-like substances, and plant extracts, has promising potential for controlling pathogen growth in food systems in response to an increased pressure from consumers to purchase more "natural-green" processed products (Burt, 2004; Butkhot et al., 2019a).

Nisin is a lanthionine-containing peptide produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Ettayebi et al., 2000). It is thermally stable and easily degraded in digestive system. Despite being granted as "generally regarded as safe" for use as food additive, such limitation of nisin in many food industries includes its expensiveness, especially when high concentration is required to gain satisfactory inhibitory activity against food-borne pathogens (Ettayebi et al., 2000). In addition, its use as biopreservative in commercial foods is compromised by the lack of inhibitory actions against Gram negative bacteria and development of nisin-resistant strains of various pathogens (Zhou et al., 2014). The problems could be overcome by the administration of nisin together with other natural substances having a synergistic action. Several authors claimed that the inhibitory spectrum of nisin in food systems can be potentiated to Gram negative bacteria with the presence of plant-derived extracts and other bioactive compounds (Govaris et al., 2010; Turgis et al., 2012; Shahbazi, 2015; Shahbazi et al., 2016). In our recent study, bacteriocin produced by *Bacillus velezensis* BUU004 demonstrated thermoresistance, stability under wide range of pH, very low cytotoxicity, and broad-spectrum antibacterial activity against important food-associated pathogens *in vitro* and food system studies, thereby having a promising potential as natural preservative in food industry (Butkhot et al., 2019a; 2019b).

The use of plant essential oils and extracts as biopresevative to inhibit growth of pathogenic and food-spoilage bacteria is of great interest in dried seafood industry owing to harmless side effects, broad spectrum of antibacterial activity, economic benefits and a long history of safe use in foods (Nazarizadeh et al., 2013). However, high concentration of plant extracts/essential oils required to achieve satisfactory inhibitory effects in food matrix as observed in *in vitro* experiment may be problematic because of degenerative sensorial quality of food products and unacceptability of consumers (Burt, 2004). Undesirable consequences of herb extracts/ essential oil supplement in foods could be obviated simultaneously with securing antibacterial efficacy by the combined addition of antimicrobial peptides. Until now, antibacterial activity of nisin and/or other bacteriocins in combination with Thai herb extracts against food-borne and spoilage bacteria in processed seafood products have not been investigated. Therefore, this study aim to compile the literatures focused on bacteriological quality of processed squid-based products, evaluate bacterial contamination in multiple steps related to dried seasoned squid preparation and storage, and extend the antibacterial efficacy of bacteriocin produced by *B. velezensis* BUU004 by combination with nisin and mixture of hot pepper and lemongrass extracts for controlling growth of contaminated bacteria in dried seasoned and crushed squid.

MATERIALS AND METHODS

Bacteriological quality of traditional processed squid

A bacteriological quality of a variety recipe of traditional ready-to-eat squid sold in Chon Buri province, Thailand was compiled from several sources as follows: authoritative collections of online journals, books, and research resources, e.g. Pubmed, Sciencedirect, Wiley online library, SpringerLink, ThaiJO, and Google scholar; manual compilation of published and unpublished data from the library of Burapha University; and research articles, review articles, academic reports, conference proceedings, and thesis from Thai Library Network (cooperation network among university libraries in Thailand). Keywords used for searching the targeted data in all available collections were viable bacteria, heterotrophic bacteria, bacteriological quality, biosafety, seafood products, processed squids, ready-to-eat squids, and Chon Buri. The criteria used for selection of articles were the findings focused on bacteriological quality in ready-to-eat and processed traditional squid products in Chon Buri province, Thailand, and published or unpublished articles in Thai and/or English before 2020. Total viable counts (TVC) in the squid product samples were extracted from each article and compared to the allowable values imposed by food administration agencies of Thailand, USA, UK, Japan and China, and related international authorities to indicate biosafety quality of the Thai traditional processed seafood products (China Food and Drug Administration, 2015; Thailand Department of Fisheries, 2011; International Organization for Standardization ISO 4833-1, 2013; Japan Department of Food Safety, 2014; UK Health Protection Agency, 2017; US Food and Drug Administration, 2011).

Bacterial contamination during preparation steps of dried seasoned and crushed squid

Dried seasoned and crushed squid was prepared in a small household facility located in a fishing village in Chon Buri province, Thailand, to imitate a real situation of the dried squid production in Thailand. Briefly, fresh splendid squids (*Loligo duvauceli*) were

Figure 1. Dried seasoned and crushed squid during multistep related to preparation and storage processes. (a) cut, cleaned and rinsed squid, (b) marinated squid in brown seasoning sauce, (c) 1st sun-dried squid, (d) squid grilled on an electric grill with periodically flipping over, (e) squid being crushed using a rolling machine, and (f) dried seasoned, and crushed squid.

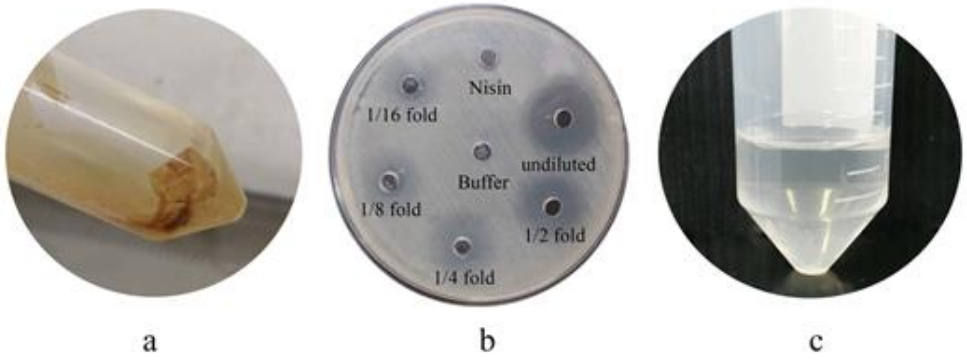

Figure 2. (a) Precipitated protein obtained from the culture of *B. velezensis* BUU004, (b) inhibition zones of the PPS-BV at different concentrations against *B. cereus* TISTR 687 using an agar well diffusion method for calculation of bacteriocin activity, and (c) the PPS-BV (800 AU/mL) dissolved in 50 mM sodium phosphate buffer.

purchased from a local market in Chon Buri province, Thailand. Medium-sized squids (15-18 cm long) were cut longitudinally in the center of the abdomen using a sharp knife. Then, manual cleaning of the squid was performed by thoroughly removing head, tentacles, skin, eyes, soft shell, beak and internal organs using tap water (Figure 1a). The 5-kg rinsed squids were marinated in brown seasoning sauce composed of chili sauce (1.5 L), sugar (500 g), chili pepper powder (80 g), chopped garlic (10 g), and vinegar (50 mL) for 1 h (Figure 1b). Subsequently, flattened squids were laid on a steel wire grate, and sun-dried from 8 am to 4 pm for two successive days (Figure 1c). After approximately 2 min grilling on an electric grill (Figure 1d), the squids were individually crushed using a rolling machine (Figure 1e) and sun-dried again until dry. Dried seasoned and crushed squids (Figure 1f) were packed into a plastic bag and stored at room temperature for 25 days. During multistep related to preparation and storage, random sampling was taken in rinsed squid, marinated squid, first sun-dried squid, grilled squid, crushed squid, second sun-dried squid, 10-day stored squid, and 25-day stored squid to evaluate bacterial quality and abundance.

Bacteriological quality was evaluated based on a protocol recommended by U.S. Food Drug Administration (1998) with some modifications. A portion (50 g) of samples collected at defined intervals was homogenized with Butterfield's phosphate-buffered water (450 mL) using a stomacher for 2 min. To enumerate TVC, homogenate was 10-fold diluted in same solution and an aliquot (0.1 mL) of each dilution was spread-plated onto Plate Count Agar (Becton BD, Sparks, Maryland, USA). All petri dishes were incubated at 35 ± 2 ºC for 24 h. All colonies were counted and expressed as colony forming unit (CFU) per g of sample. Bacterial identification was conducted following a basis of Gram-staining, cell morphology, and biochemical characteristics recommended by Winn et al. (2006) and API test kits (bioMerieux, Marcy l' Etoile, France). All measurements were achieved in triplicate.

Preparation of partially-purified solution containing bacteriocin from *B. velezensis* BUU004 (PPS-BV)

The partially-purified solution containing bacteriocin was obtained from the culture of *B. velezensis* BUU004 (Butkhot et al., 2019a). The strain was grown in a 250-mL flask containing 100 mL of Trypticase Soy Broth (Becton BD) and incubated at 30°C, 200 rpm for 18 h in a shaking incubator. Cell suspension was centrifuged at 8,000 *g*, 4°C for 10 min. The supernatant containing bacteriocin was collected, and then added with ammonium sulfate at 80% saturation to precipitate proteins with gentle stirring overnight at 4°C. Protein precipitate was harvested by centrifuging at 10,000 *g* at 4°C for 30 min (Figure 2a), re-suspended in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed using a dialysis membrane

tubing (1 kDa cutoff, Spectrum Laboratory, New Brunswick, New Jersey, USA) at 4 °C overnight. Following passing through a 0.45 μm syringe filter (Whatman, Kent, UK), the PPS-BV was tested for bacteriocin activity in arbitrary unit (AU) against *Bacillus cereus* TISTR 687 using an agar well diffusion method (Figure 2b; Butkhot et al., 2019b). The PPS-BV (800 AU/mL) was prepared by dissolving in 50 mM sodium phosphate buffer (Figure 2c) and frozen at -20 °C until use.

Herb extraction

Lemongrass stems (*Cymbopogon citratus* (DC) Stapf.) and hot pepper fruits (*Capsicum frutescens* L.) were purchased from a local spice store in Chon Buri province, Thailand. Herb preparation and extraction were followed a protocol described by Soodsawaeng et al. (2021a). The herbs were chopped using a table knife, dried in a plant drier at 35 °C for 72 h, and then ground using an electronic blender. The dried herb powders were individually submerged with 95% ethanol at a ratio of 1:10 of material to extractant, and agitated in a shaking incubator (JSR, JSSI-100C model, Cheongju, South Korea) at 120 rpm, 30 °C for 72 h. The supernatants were vacuum-filtered using a Whatman filter membrane no.1 and evaporated at 40°C and 175 mbar, using a rotary evaporator (Buchi R-215, Flawil, Switzerland). The ethanolic extract stock was made, sterilized using a 0.45 μm syringe filter, and stored in an amber bottle at -20°C.

Nisin preparation

Nisin contained 2.5% active nisin with minimum potency of 10^6 International Units (IU/g) was purchased from Sigma-Aldrich Chemical Co, Darmstadt, Germany. Nisin solution was prepared by dissolving nisin powder (100 mg) in 0.02 N HCl, adding with sterile distilled water to produce a final concentration of 10^3 IU/mL, filtering using a 0.45 μm syringe filter, and storing in a 4°C refrigerator.

Synergistic study of the PPS-BV in combination with nisin and the mixed herb extracts on viable bacteria in dried seasoned and crushed squid

Antibacterial efficacy of the PPS-BV in combination with nisin and the mixed herb extracts on viable bacterial count was examined according to the method described by Butkhot et al. (2019a). Due to having a significantly increased TVC compared to other stages, dried seasoned and crushed squid during a storage period was used as a food model in this experiment. The squids were produced following procedures mentioned earlier and stored in a plastic bag at 4 ºC for 28 days. A 2 x 2 cm piece of the squid was made using a sterile scissors. Treatment of the samples included supplementation of 1) sterile distilled water (control), 2) nisin (10^3 IU/mL), and 3) a combined additive of the PPS-BV (800 AU/mL), nisin (10^3 IU/mL) and the mixture of herb extracts (160 mg/mL). A minute volume (0.1 mL) of the supplements was introduced onto whole surface of the squid samples following the respective treatments. After air-drying in a biosafety cabinet for 15 min, the sample in each treatment was placed in a sterile plastic bag (3 x 5 inches; 1 sample: 1 bag) to prevent cross contamination, and then stored in a 4ºC refrigerator. Enumeration of viable bacteria in the squid samples was conducted at 15 min, 1, 7, 14, 21 and 28 days post-inoculation using a spread plate technique as aforementioned. TVC of the control and treated groups was compared to the allowable limits imposed by food administration agencies of Thailand, USA, UK, Japan and China, and related international authorities as mentioned previously to evaluate the efficacy of the tested additives.

Data analysis

Data are represented as mean ± standard deviation (S.D.). The numbers of bacteria were 10-log transformed to normalize distribution when needed prior to statistical analyses. Differences were analyzed using a One-way ANOVA following the Tukey's multiple comparison test at a significant level of $P < 0.05$. All statistical analyses were performed using a Minitab Version 18.1.0.

RESULTS

Bacteriological quality of processed squid products distributed in Chon Buri province, Thailand based on literatures

TVC of traditional processed squid complied from literatures was in the ranges of 3.0×10^2 to 5.2×10^9 CFU/g (Figure 3). Of all samples compiled, 27.0% (10/37), 40.5% (15/37), 51.4% (19/37) and 86.5% (32/37) samples contained TVC over the maximum limits acceptable for ready-to-eat fishery products set by US Food and Drug Administration (2011; 5×10^5 CFU/g), UK Health Protection Agency (2017) and ISO 4833-1 (2013; 1×10^5 CFU/g), Thailand Department of Fisheries (2011) and Japan Department of Food Safety (2014; 5×10^4 CFU/g), and China Food and Drug Administration (2015; 3×10^3 CFU/g), respectively (Figure 3). Overall, a public health issue of traditional processed squid distributed in Chon Buri province, Thailand is alarming due to 86.5% samples compiled between 2002 and 2019, containing TVC over the acceptable value of at least one criterion imposed by food administration agencies.

Bacterial abundance during various steps related to production of dried seasoned and crushed squid

Squid samples contained low TVC in the ranges of $1.2 \pm 0.7 \times 10^2$ to $2.6 \pm 0.9 \times 10^2$ CFU/g during the steps between rinsing and second sun-drying (Figure 4a). In post-prepared products, TVC appeared to increase significantly during 25 days of storage at room temperature. TVC increased significantly to $2.0 \pm 0.2 \times 10^3$ CFU/g at 10 days of storage and $2.1 \pm 0.5 \times 10^3$ CFU/g at 25 days of storage (Figure 4a).

Bacterial composition of squid samples changed towards a multistep of production and storage. In rinsed squid, six bacterial species were isolated including *Bacillus pasteurii* (30.8%), *Micrococcus luteus* (15.4%), *B. brevis* (15.4%), *B. badius* (15.4%), *Bordetella parapertussis* (15.4%), and *B. insolitus* (7.6%; Figure 4b). In marinated squid, bacterial component was changed to *Staphylococcus carnosus* subsp. *utilis* (63.6%), *B. pasteurii* (18.2%), *S. piscifermentans* (9.1%) and *B. badius* (9.1%). Thereafter, isolated bacteria increased to eight species belonging to *S. piscifermentans*, *M. luteus*, *B. pasteurii*, *B. brevis*, *B. firmus*, *B. pantothenticus*, *B. insolitus*, and *Bo. parapertussis* in first sun-dried squid.

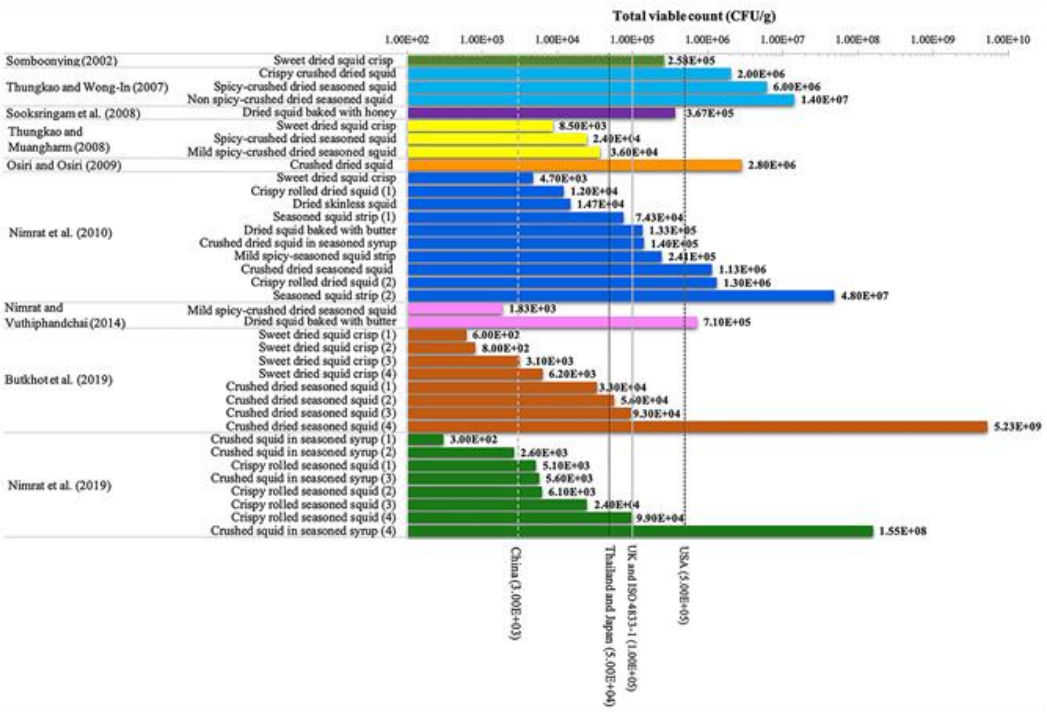

Figure 3. Viable bacterial count of ready-to-eat squid products sold in a local market in Chon Buri province, Thailand compiled from various literatures, compared to the bacteriological quality standards imposed by food administration agencies of several countries and related international authorities. Acceptable limits of bacterial count in cooked ready-to-eat products are within 5.0 x 10^4 CFU/g by Thailand Department of Fisheries (2011) and Japan Department of Food Safety (2014), 5.0 x 10^5 CFU/g by US Food and Drug Administration (2011), 1.0 x 10^5 CFU/g by UK Health Protection Agency (2017) and ISO 4833-1 (2013), and 3.0 x 10^3 CFU/g by China Food and Drug Administration (2015).

Bacillus species (*B. pasteurii*, *B. brevis*, and *B. insolitus*) were predominantly isolated in squid during the grilling and crushing steps. Then, two *Bacillus* species, such as *B. pasteurii* and *B. insolitus*, were present in the 2nd sun-dried squid. At 25 days of storage, eight bacterial isolates were recovered with the main bacterial species of *S. piscifermentans* (48.2%), and *B. pasteurii* (19.2%; Figure 4b).

Efficacy of the PPS-BV in combination with nisin and the mixed herb extracts against TVC in dried seasoned and crushed squid

High TVC ranging from 4.27 ± 0.33 × 10^3 to 1.37 ± 0.02 × 10^4 CFU/g was observed in the control squids during 28-day trial. At 15-min post-exposure, the lowest TVC was produced with the presence of the PPS-BV in combination with nisin and the mixed herb extracts in dried seasoned and crushed squid (1.75 ± 0.43 × 10^2 CFU/g) and, then TVC remained in the range of 2.33 ± 0.82 × 10^2 to 1.53 ± 0.11 × 10^3 CFU/g and dropped to 7.50 ± 4.33 × 10^1 CFU/g at the end of experiment. TVC in squid samples introduced with nisin was also significantly different (P < 0.05), compared to the control, but significantly (P < 0.05) higher than that of the dried squid added with the PPS-BV plus nisin and the mixed herb extracts during 28-day refrigerated storage, except at day 7 and 14 of storage (Figure 5).

In comparison with the acceptable values, TVC in the control and the dried squid introduced with nisin during 28-day refrigerated storage met the standards for processed seafood products announced by Thailand Department of Fisheries (2011), ISO 4833-1 (2013), Japan Department of Food Safety (2014), UK Health Protection Agency (2017) and US Food and Drug Administration (2011), except China Food and Drug Administration (2015). Interestingly, TVC in the squid samples added with a combination of the PPS-BV with nisin and the mixed herb extracts was within allowable limit of all criteria throughout 28-day trial (Figure 5).

DISCUSSION

Bacteriological quality and bacterial abundance during multi-steps related to prepared and post-prepared dried seasoned squid

Deteriorated bacteriological quality of food products is a

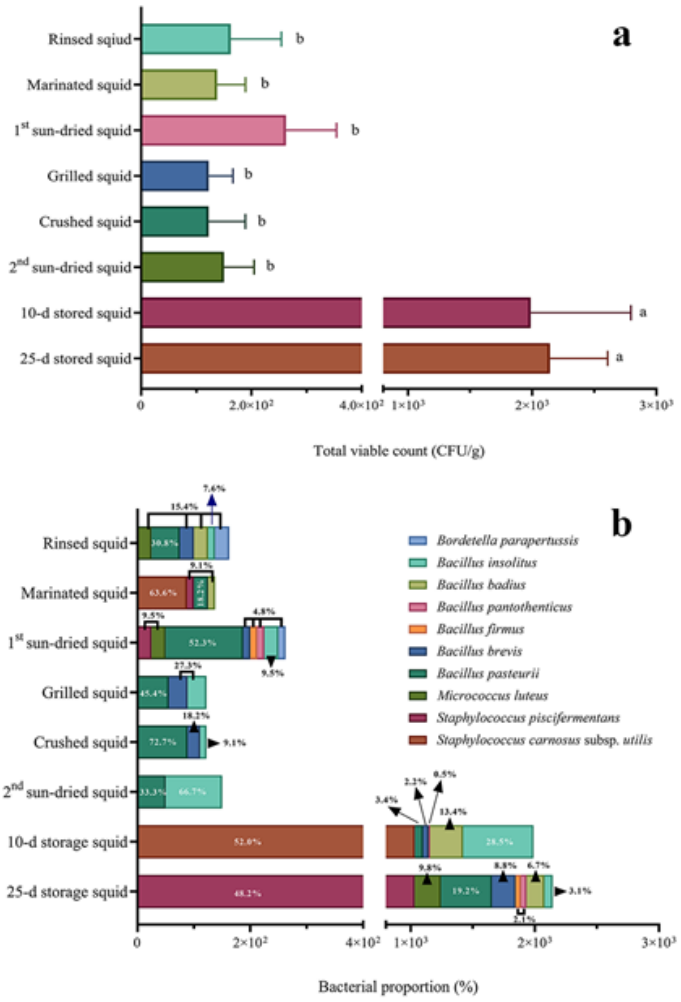

Figure 4. Total viable count (a) and proportion of isolated bacteria (b) in dried seasoned and crushed squid during multistep related to preparation and storage. Bars with letters denote significant difference ($P < 0.05$).

serious safety issue. Although the growth of most microorganisms can be limited by high salt and low moisture contents, almost all processed squid products (86.5%) compiled from the literatures contained TVC over the allowable limits declared by Thailand and international food administration agencies. This suggested that a health hazard from consumption of traditional processed squid products distributed in Chon Buri, province, Thailand is indeed alarming. In general, several factors throughout all stages of the food production and distribution systems affect food safety. Food-borne pathogenic and spoilage bacteria can enter food products during: primary production (in the farm/sea where animals are raised or caught); transportation; food processing; storage; distribution; and preparation and serving (both outside and inside the home; Bintsis, 2018). Thus, investigations of bacteriological quality involved production of dried seafood products would assist in identifying certain sources of contamination. In this study, predominant bacteria isolated along a multistep production of dried seasoned and crushed squid were *Bacillus, Staphylococcus*, and *Micrococcus*. Similarly, Butkhot et al. (2019a) reported that dried seafood products were composed mainly of several species in the genera of *Staphylococcus*, *Bacillus*, and *Pantoea.* Due to their water and environmental origins, the presence of *Bacillus, Micrococcus* and *Bordetella* as dominant species in post-rinsed squid is possibly associated with poor personnel hygiene and inadequate handling (Moon et al., 2017). In the next step, bacterial abundance in the squid marinated in brown seasoning sauce was changed to *S. carnosus* subsp. *utilis* and *S. piscifermentans* and a few species of *Bacillus* remained survived, namely *B. pasteurii* and *B. badius*. The shifting of bacterial composition is perhaps expected due to antimicrobial feedback of spices, such as chili pepper, garlic and other additives, used as ingredients of brown seasoning sauce. Multiple bioactive phytochemicals present in garlic and

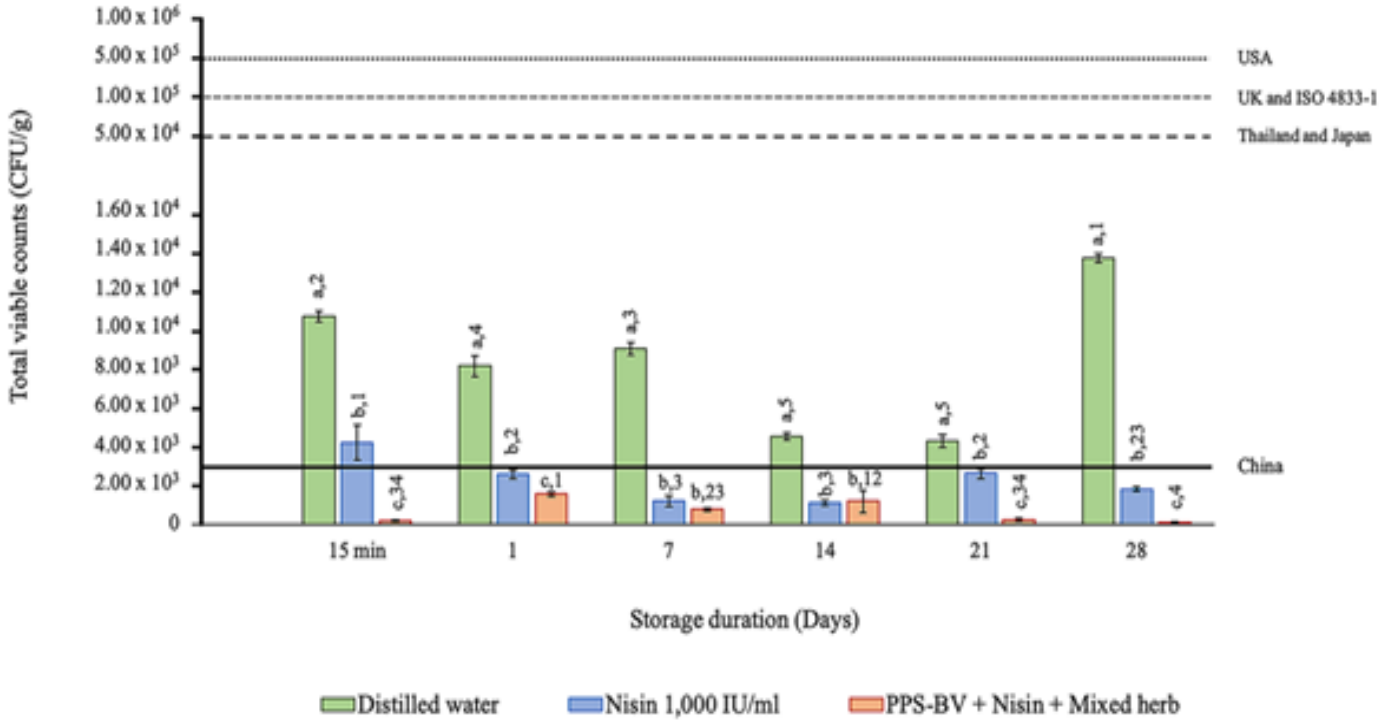

Figure 5. Effect of nisin, and the PPS-BV in combination with nisin and the mixed herb extracts on total viable counts of dried seasoned and crushed squid during 28 days of refrigerated storage, compared to the allowable limits imposed by food administration agencies of several countries and related international authorities. Bars with letters at each sampling period denote significant difference ($P < 0.05$) among treatments. Bars with numbers within the same treatment denote significant difference ($P < 0.05$) over the time. The acceptable limits of ready-to-eat fishery products are within 5.0 x 10^4 CFU/g by Thailand Department of Fisheries (2011) and Japan Department of Food Safety (2014), 5.0 x 10^5 CFU/g by US Food and Drug Administration (2011), 1.0 x 10^5 CFU/g by UK Health Protection Agency (2017) and ISO 4833-1 (2013), and 3.0 x 10^3 CFU/g by China Food and Drug Administration (2015).

chili pepper have been reported to have a strong antibacterial potential against *Aeromonas*, *Bacillus*, *Citrobacter*, *Clostridium*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Mycobacterium*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Listeria*, and *Vibrio* (Shokrzadeh and Ebadi, 2006; Omolo et al., 2014). However, re-emergence of several species belonging to *Bacillus*, *Staphylococcus*, *Micrococcus* and *Bordetella* was observed in 1st sun-dried products in this study. In Thailand, the operation and processing conditions of traditional dried seafood products in small factories or household facilities are primitive with rudimentary hygienic practices (Butkhot et al., 2019a). The facilities have free access of biotic and non-biotic agents, such as pathogens, flies, insects, rodents, dust, soil, and hazardous chemicals and pollutants into the products. These reasons may account for a deteriorative bacteriological quality of the traditional Thai squid products observed in this study. Interestingly, viable bacterial count in dried squid products appeared to significantly increase during storage and distribution periods for 25 days in this study. Such a phenomenon may reflect a poor handling practice of post-prepared products supported by the presence of *S. carnosus* subsp. *utilis* and *S. piscifermentans* as the predominant flora due to their human skin origin (Becker et al., 2014). In retail stores in Thailand, the products are usually stored at room temperature and handled without suitable hygienic awareness during being portioned from bulk containers into small plastic bags for sale (Butkhot et al., 2019a). Of all bacteria isolated, *Bo. parapertussis* causes a pertussis-like syndrome in human (Masin et al., 2015). *M. luteus is considered as an opportunistic pathogen associated with* meningitis, septic arthritis, endocarditis, chronic cutaneous infections and catheter infections in immunocompromised patients (Becker and von Eiff, 2011).

In order to minimize the risk of microbial contamination and secure food safety from farm to fork of the traditional Thai squid products, the factories should follow the microbiological guidance, such as. Good Hygiene Practices (GHPs), Good Manufacturing Practices (GMPs) and Hazard Analysis Critical Control Point (HACCP) systems developed by World Health Organization and United States Food and Drug Administration. Stakeholders, such as government and food industry should promote education programs based on basic

principles for microbiological food safety to create biosafety awareness of the food personnel and consumers. In addition, due to a markedly increased TVC in dried seasoned squids during storage and distribution processes observed in this study, it is imperative to implement an effective technique for minimizing the bacterial growth in the products to avoid the outbreak of food-borne illnesses.

Antibacterial efficacy of the PPS-BV with the presence of nisin and the mixture of herb extracts

Safety issue of chemical preservatives has been questioned by consumers in terms of the potential carcinogenic and toxic effects on human health. In our recent study, the PPS-BV was inactive to control the growth of spoilage bacteria in the dried seasoned squid (Soodsawaeng et al., 2021b). We postulated that such a phenomenon may be due to easy degradation of bacteriocin by indigenous and/or microbial proteolytic enzymes, its interactions with the food components, like proteins, carbohydrates and fats in squid, and biofilm formation of bacterial flora. Likewise, the mixed herb extracts from hot pepper and lemongrass demonstrated low inhibitory activity against food spoilage bacteria in food system trial (Soodsawaeng et al., 2021b). Therefore, in the present study, we introduced the PPS-BV in combination with nisin and the mixed herb extracts into dried seasoned and crushed squid during storage and distribution processes. The novel combination represented a great potential as biopreservative supported by a significant decrease in TVC in squid samples to below allowable limits of all food administration agencies during 28 days of chilled storage. Our results are similar to several previous reports. Field et al. (2015) reported that a solution containing a bioengineered derivative nisin V in combination with low concentration of either carvacrol or trans-cinnamaldehyde was synergistically effective to delay lag phase and markedly reduce viable cells of *L. monocytogenes* in laboratory media, chocolate milk drink and chicken noodle soup. Bag and Chattopadhyay (2017) claimed that the activity of nisin was significantly enhanced against planktonic cells and biofilm formation of food-borne pathogenic *B. cereus* and *S.* Typhimurium when nisin was combined with *p*-coumaric acid. Similarly, administration of nisin simultaneously with essential oil (0.1-0.2%) from *Ziziphora clinopodioides* resulted in a significant decrease in *E. coli* O157:H7 count in raw beef patty during 9 days of refrigerated storage (Shahbazi et al., 2016), and complete mortality of *E. coli* O157:H7 in doogh yoghurt drink (Shahbazi, 2015). Antilisterial activity of enterocin AS-48, a cyclic bacteriocin produced by *Enterococcus feacalis*, was significantly extended by combined addition of one of these essential oils (thyme verbena, thyme red, Spanish oregano, ajowan, tea tree, clove, and sage) into the Russian type salad (Molinos et al., 2009).

A significant reduction in TVC of the squid samples introduced with the PPS-BV in combination with nisin and the mixed herb extracts in this study is likely to involve in a synergistic activity between the components in the combined additives. Lemongrass extract/essential oil has been reported to contain citral chemotype: geranial, neral and limonene with a wide inhibitory spectrum against pathogenic *E. coli* O157:H7, *Salmonella* Typhimurium, *S. aureus* and *L. monocytogenes* (Oussalah et al., 2007). Cinnamic acid and *m*-coumaric acid are predominantly present in chili pepper extract contributing to the inhibition of food-borne pathogens (Dorantes et al, 2000). A recent study revealed the bacteriocin produced by *B. velezensis* BUU004 capable of killing food-borne pathogens through membrane pore formation (Butkhot et al., 2019a). The actual mode of mechanisms of the bacteriocins combined with nisin and naturally herb-based substances is not fully understood. Ettayebi et al. (2000) observed the complete mortality of *L. monocytogenes* and *B. subtilis* by a combination of sub-inhibitory concentrations of thymol, a major active component of thyme, and nisin Z. They hypothesized that the bacterial death might be due to intracellular metabolite dissipation caused by changes in the cytoplasmic membrane structure and permeability, and the leakage and/or passage of a variety of molecules and ions. Hyldgaard et al. (2012) also pointed out the bactericidal effects of the combined agents thought to be associated with destabilization of the bacterial membrane structure by attacking the cytoplasmic membrane, enhancing the uptake the antimicrobial peptides, changing in the proton motive force, and inhibiting enzymatic systems. Similarly, we observed that the PPS-BV acted synergistically with the mixture of lemongrass and hot pepper extracts against pathogenic *E. coli* ATCC 25922 and *S.* Typhimurium TISTR 292 evident from formation of the membrane pores and cell lysis (Soodsawaeng et al., 2021a). An increased antibacterial efficacy of the combined additives observed in this study may not be arisen from only one factor. Therefore, additional future studies are needed to identify the active constituents present in the combined additive and elucidate their mechanisms of the antimicrobial actions. This would be useful for technological applications in food industry. This study may spotlight in the field of study to use the bacteriocins simultaneously with naturally plant-based substances as biopreservative in dried seafood products. Combinatorial strategy of bacteriocins and plant-derived components can broaden antibacterial spectra, enhance quality of processed seafood products, and reduce the potential adverse hazards of synthetic counterparts. It also has financial benefits by reducing the costs of treatment due to an expensive commercial preservative.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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RAPD-based evaluation revealed genetically diverse populations of *Pseudomonas aeruginosa* and *Staphylococcus aureus* lytic bacteriophages isolated in urban sewage and Ebrie Lagoon, Côte d'Ivoire

Audrey A. Addablah[1,2*], Solange Kakou-Ngazoa[1], Eric E. Akpa[2], Emmanuella Adioumani[1,2], Fred M'Bourou Ndombi[1], Serge Aoussi[1] and Mireille Dosso[1]

[1]Plateforme de Biologie Moléculaire, Institut Pasteur de Côte d'Ivoire, 01 BP 490 Abidjan 01, Côte d'Ivoire.
[2]Laboratoire de Pharmacodynamie Biochimique, Université Félix Houphouët-Boigny, 01 BP V34 Abidjan 01, Côte d'Ivoire.

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The application of bacteriophages biocontrol requires the formulation of genetically distinct bacteriophages in a phage cocktail. Random Amplified Polymorphic DNA (RAPD) - PCR is considered a cheap, reproducible, and readily applicable tool in detecting phage diversity compared to other molecular techniques such as whole-genome sequencing. We used in this study the RAPD-PCR technique to assess the genetic diversity of 28 bacteriophages infecting *Pseudomonas aeruginosa* and *Staphylococcus aureus*. According to their RAPD profiles, isolated phages were grouped into 2 main clusters which included phages from the same host. The typing by RAPD-PCR of newly isolated phages was useful to assess the genetic diversity bypassing previous whole-genome sequencing analysis. These genetically distinct phages lytic against *P. aeruginosa* and *S. aureus* could potentially be used in a phage cocktail for biocontrol against these clinically and industrially relevant bacteria.

Key words: Phages, genetic diversity, RAPD PCR, *P. aeruginosa*, *S. aureus.*

INTRODUCTION

Bacteriophages (phages), the viruses of bacteria are the most abundant biological entities in the biosphere and are always associated with their host bacteria in the same ecological niche. Consequently, phages have an impact on the bacterial ecosystems both locally in our immediate communities and globally (Abedon, 2008; de Leeuw et al., 2020; Harada et al., 2018). Generally, both *Pseudomonas aeruginosa* and *Staphylococcus aureus* are virulent pathogenic organisms for many clinical conditions. *P. aeruginosa* is one of the major life-threatening opportunistic bacteria responsible for nosocomial infections in immunocom promised people, a leading cause of lung infections and ventilator-associated pneumonia (Essoh et al., 2015; Wang et al., 2017), while

*Corresponding author. Email: addablah.audrey@gmail.com.

S. aureus is responsible for skin and soft tissues infections and invasive diseases such as endocarditis, osteomyelitis (Wang et al., 2016). Furthermore, both species are notorious for their resistance to antimicrobials. *P. aeruginosa* and *S. aureus* are members of the ESKAPE pathogens which are known for widespread resistance to almost all classes of antimicrobials used in a hospital setting (Santajit and Indrawattana, 2016). Due to the importance of these infections worldwide, phages' activity against these pathogens were considered as an eco-friendly alternative to antibiotics (Abatángelo et al., 2017; Essoh et al., 2013; Kifelew et al., 2020; Oliveira et al., 2015; Song et al., 2021). Phages with lytic activity against these bacterial pathogens are sourced from the environment or clinical specimens linked to diseases caused by *P. aeruginosa* and *S. aureus*.

Bacteriophages have a narrow host range as a single phage may infect only specific strains. However, some phages are able to infect various bacterial species (Hamdi et al., 2017). The narrow host range of phages has not limited their use in industries and therapeutically. This nature and the ability of bacteria to evolve to resist a lytic phage, therefore, necessitate the use of phage cocktails in biocontrol. However, one of the best methods for the assessment of genetic diversity is whole-genome sequencing (WGS) and analysis. These techniques are expensive and not always available in some countries. Thus, a reproducible and affordable approach would be very valuable to readily assess diversity among newly isolated phages. Random amplified polymorphic DNA (RAPD)-PCR has been used to discriminate among different phage lineage and for assessment of their genotypic diversity in terms of hours (Ács et al., 2020).

In this study, we investigate lytic phages against *P. aeruginosa* and *S. aureus* specific-phages. The phage isolation was performed with samples collected from urban sewage and Ebrié Lagoon of Abidjan, Côte d'Ivoire, given that sewage and environmental water are optimal sources of phages (Alharbi and Ziadi, 2021; Weber-Dąbrowska et al., 2016). The RAPD PCR of isolated phage DNA extracts helped to discriminate between the phages and to assess their genetic diversity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two bacterial strains isolated in 2017 were used for the phage isolation. *P. aeruginosa* strain "P02" was isolated in sewage sample and *S. aureus* strain "S04" in street vendor hands. Both strains were provided by Plateforme de biologie moléculaire of Institut Pasteur de Côte d'Ivoire. The strains were grown overnight in Lysogeny Broth (LB) (10 $g.l^{-1}$ NaCl, 10 $g.l^{-1}$ Tryptone, 5 $g.l^{-1}$ Yeast Extract) and stored in 200 µl aliquots in medium with 30% glycerol at -20°C. A hundred microliters from stored bacteria were added to 3 ml of LB and grown overnight at 37°C for isolation purposes.

Phage isolation and purification

Fifteen samples of urban sewage were collected from wastewater collectors of the municipality of Yopougon, Abidjan. Fifteen other samples were collected in the Ebrié Lagoon of Abidjan. Samples were collected using sterile 500-ml glass vials, connected to a rope. Biosafety rules were applied for the protection of the sampler and the samples (Personal protective equipment). Samples were taken instantaneously by immersing the vial in the effluent to be sampled. The opening of the vial was placed facing the effluent stream, under the surface of the liquid, and a 300 ml sample was taken. A space of 2.5 cm is left between the level of the sample and the lid of the bottle. The vial is sealed and kept in a container at 4°C for transport to the laboratory.

The samples were then enriched to detect *P. aeruginosa* and *S. aureus* lytic phages according to a modification of (Van Twest and Kropinski, 2009) procedure. Briefly, samples were centrifuged at 12000 g for 10 min and filtered through 0.22 µm pore membranes. Ten milliliters of filtered water were added to 10 ml of double-strength LB broth (10 $g.l^{-1}$ NaCl, 10 $g.l^{-1}$ Tryptone and 5 $g.l^{-1}$ Yeast Extract) with 100 µl of the enrichment bacterial strain. The mixture was incubated at 37°C, 48 h. Thereafter, the culture was centrifuged at 12000 g, 5 min, and the supernatant was filtered and evaluated for the presence of lytic phages using the double-layer agar method. Serial decimal dilution up to 10^{-8} of filtered lysate was carried out. Then, 100 µl of overnight bacterial culture (18 – 24 h) was mixed with 0.6% soft LB agar and poured on a 1.5% bottom LB agar plate. When the top agar layer was set, serial dilutions of filtered lysate were spotted onto the overlay, plates were incubated overnight at 37°C and observed for isolated plaques. Phage plaques were purified three times to ensure phage purity (Salem et al., 2015). A sterile truncated tip was stabbed through a single well-isolated plaque that was immediately added to 500 µl of SM buffer (50 mM Tris-HCl pH7.5, 100 mM NaCl, 8.1 mM $MgSO_4$), mixed and refrigerated for 1 h at 4°C. After incubation, the mixture was centrifuged and 300 µl of supernatant was filtered and transferred to a new tube. Purified phages were preserved at 4°C before genomic DNA extraction and at -20°C for long-term conservation.

Genomic fingerprinting by RAPD analysis

Phage DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described previously (Jakočiūnė and Moodley, 2018) from 450 µL of phage stocks with a titer between 10^9 to 10^{14} plaque-forming unit (pfu)/ml. RAPD-PCR was carried out according to a modification of the method described previously (Kumari et al., 2009). Primers P1 (5'-CCGCAGCCAA-3') and P2 (5'-AACGGGCAGA-3') were used as designed in the study of Gutiérrez et al. (2011). PCR mixture (50 µl) consisted of 1 µl of phage DNA, 5.0 µl 5X Green Reaction Buffer, 5.0 µl 5X Green Reaction Buffer, 3.0 µl $MgCl_2$ (25 mM), 1.0 µl dNTP's (25 mM) (Promega, Wisconsin, USA), 1 µl primer, 0.5 µl GoTaq G2 Flexi DNA polymerase (5 U/µl) (Promega, Wisconsin, USA) and 29.5 µl DNase/RNase free water (Promega, Wisconsin, USA). Reactions were performed at 95°C for 5 min followed by 45 cycles of consecutive primers annealing (26 and 31°C for 3 min), extension (72°C for 2 min), denaturation (94°C for 1 min), and final extension (72°C for 10 min).

DNA band patterns were obtained after gel electrophoresis on 2% agarose gel of the RAPD-PCR reaction products (15 µL). Gels were run for 120 min at 80 V using a 1kb DNA ladder (Promega) as a molecular weight marker. The gel images were recorded using the Gel Doc EZ Gel Documentation System (BioRad). The RAPD profile was analyzed using the software GelJ v2.0 (Heras et al., 2015). The similarity matrix was calculated based on the Pearson

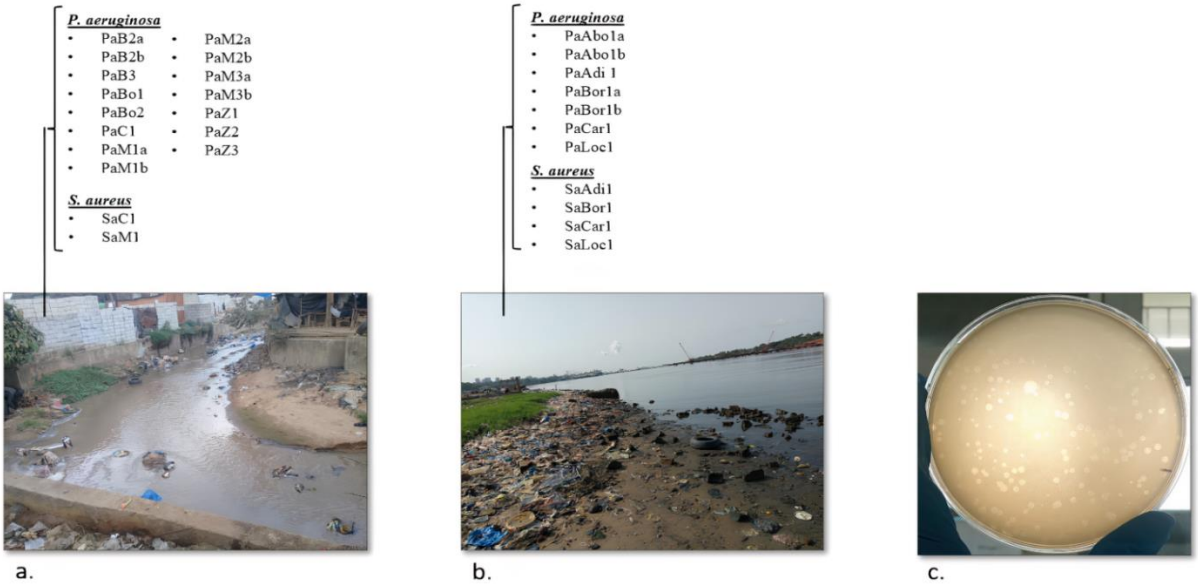

Figure 1. Phages isolated in water samples of (a.) urban sewage and (b.) Ebrié Lagoon with *P. aeruginosa* and *S. aureus* as host bacteria. (c) Plaques formed by phage SaM1 infecting *S. aureus* during the purification.

Table 1. Summary of phage isolated from water samples from urban sewage and Ebrié Lagoon.

Biotope	Site	Water samples (n)	Phages lytic for		Range of phage titre (pfu/ml)
			P. aeruginosa	*S. aureus*	
Urban sewage	1	3	6	1	$5.5\times10^{4} – 1.5\times10^{7}$
	2	3	1	1	$3.5\times10^{5} – 3.0\times10^{7}$
	3	3	2	0	$6.5\times10^{2} – 7.0\times10^{4}$
	4	3	3	0	$1.35\times10^{4} – 4.5\times10^{7}$
	5	3	3	0	$7\times10^{3} – 1.5\times10^{7}$
Ebrié Lagoon	6	3	2	1	$9\times10^{6} – 1.45\times10^{8}$
	7	3	1	1	$1.25\times10^{12} – 4.5\times10^{12}$
	8	3	1	1	$8.0\times10^{6} – 1.05\times10^{7}$
	9	3	2	0	$10^{7} – 1.45\times10^{7}$
	10	3	1	1	$1.22\times10^{5} – 9.53\times10^{6}$
Total		30	22	6	

correlation coefficient, and its corresponding dendrogram was deduced using the complete linkage clustering method in GelJ.

RESULTS

Phage collection

In this study, 28 lytic phages were isolated, 17 in urban sewage samples and 11 in Ebrie lagoon water samples (Figure 1). *P. aeruginosa* is the host of 75% of isolated phages, compared to 25% for *S. aureus*. Site 1 provided the largest number of *P. aeruginosa* specific-phages. This latter could be isolated at least once in all studied sites. On the contrary, *S. aureus*-specific-phages could not be recovered in four sites (Table 1).

Phage genetic diversity analysis

RAPD-PCR on phage DNA constitutes a suitable approach to quickly assess the genetic diversity among

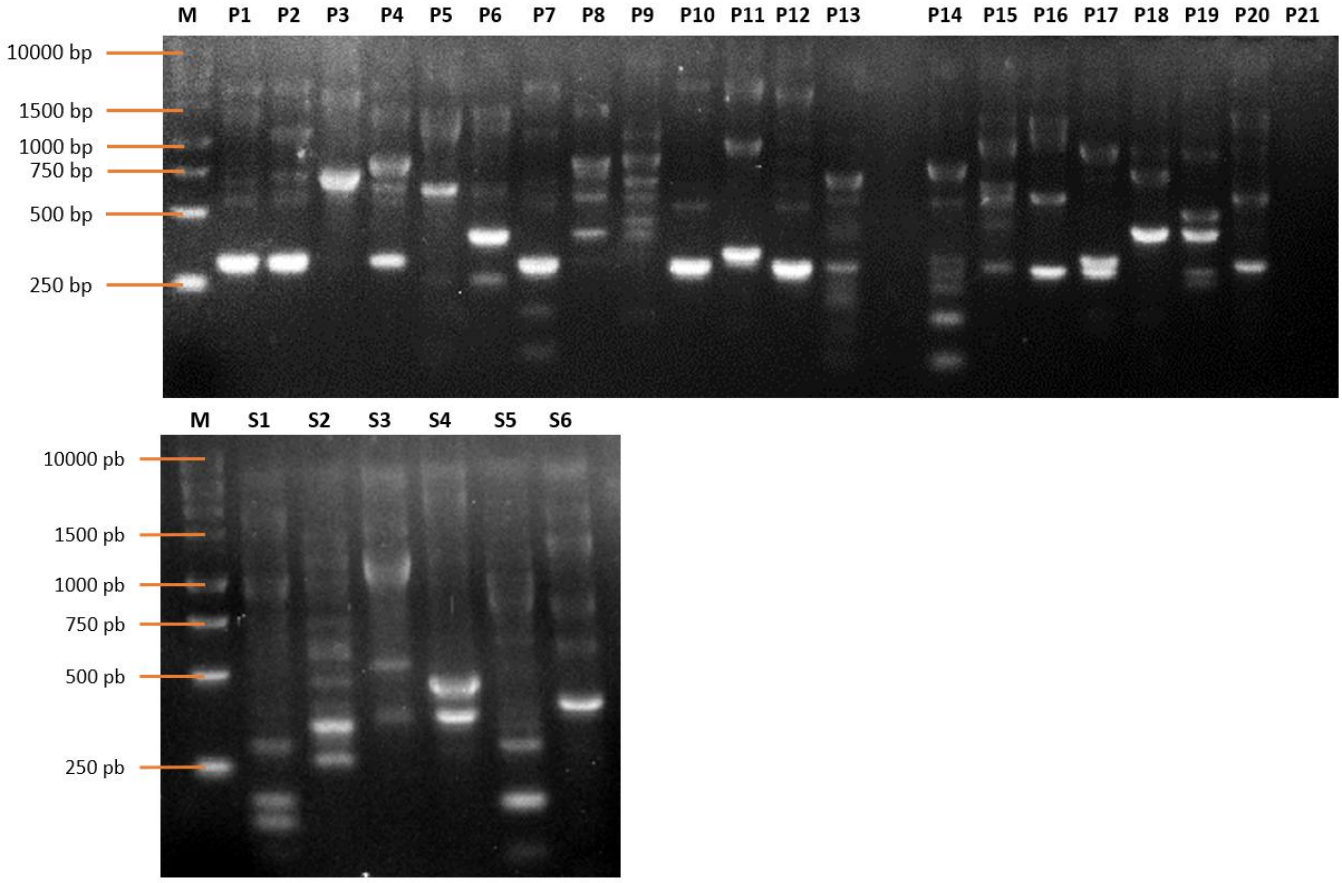

Figure 2. Agarose gel electrophoresis showing RAPD band patterns obtained from isolated phages infecting (a) *P. aeruginosa* and (b) *S. aureus*. M: DNA ladder marker 1kb; P1: PaM1a; P2: PaM1b; P3: PaM2a; P4: PaM2b; P5: PaM3a; P6: PaM3b; P7: PaBo1; P8: PaBo2; P9: PaC1; P10: PaB2a; P11: PaB2b; P12: PaB3; P13: PaZ1; P14: PaZ3; P15: PaAbo1a; P16: PaAbo1b; P17: PaAdi1; P18: PaLoc1; P19: PaCar1; P20: PaBor1a; P21: PaBor1b; S1: SaAdi1; S2: SaBor1; S3: SaCar1; S4: SaLoc1; S5: SaC1; S6: SaM1.

newly isolated bacteriophages infecting the same species. Two different primers were used, out of which only P2 (5'-AACGGGCAGA-3') provided bands with almost all the phages, except for phages PaZ1 and PaBor1b both infecting *P. aeruginosa*. No profile was observed with primer P1. The genomic fingerprints from the phages infecting the same host were likely distinct and have shown different numbers of bands ranging from 2 to 8 respectively for PaM2a and PaC1 infecting *P. aeruginosa*, and from 3 to 10 respectively for SaLoc1 and SaC1 infecting *S. aureus*. Also, the fingerprinting differed by the bands' intensity, and the fragment size varied ranging from less than 250 bp to more than 10.000 bp (Figure 2).

However, phages PaM1a, PaM1b, PaBo1, PaB2a, and PaB3 are closely related with a similarity of Pearson greater than 98% (Figure 3). They are all isolated in urban sewage samples. Unsurprisingly, the dendrogram revealed 2 main clusters: cluster A including all phages infecting *S. aureus,* and cluster B including phages infecting *P. aeruginosa*. Similarity among phages infecting *S. aureus* is more than 86%, while similarity among *P. aeruginosa* phages is 78%.

DISCUSSION

In this study, water samples from urban sewage of Yopougon and Ebrie Lagoon in Abidjan, Côte d'Ivoire were screened for the presence of *P. aeruginosa* and *S. aureus* specific lytic phages. Bacteriophages infecting *P. aeruginosa* were easily isolated from sewage as reported by previous studies conducted in urban sewage of the municipality of Cocody in Abidjan (Essoh et al., 2015). However, there are no previous documented reports of *S. aureus*-specific phages isolated in sewage and environmental water in Côte d'Ivoire. We noticed also that isolated phages are unevenly distributed regarding the sampling ecosystem and the isolation strain. Phages are known to be found where their host is present (Son et

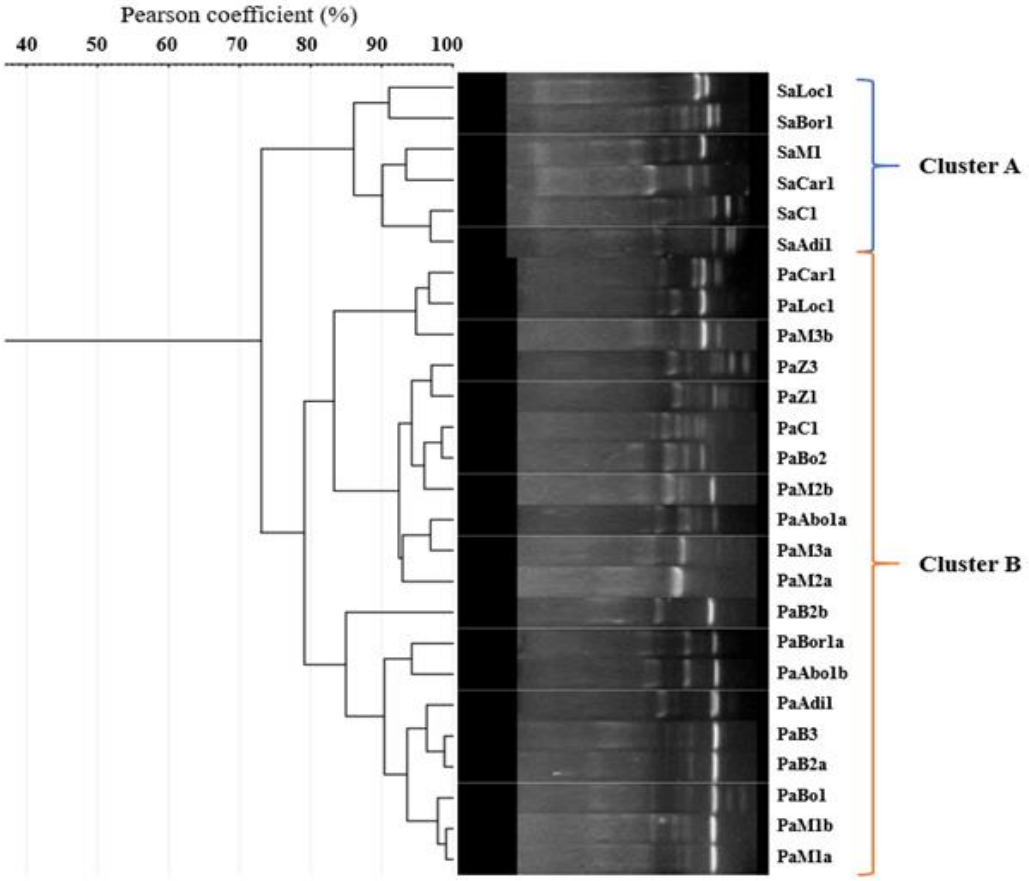

Figure 3. Dendrogram obtained after the analysis of RAPD band patterns of phages infecting *P. aeruginosa* and *S. aureus*. The similarity between samples was calculated based on the Pearson correlation coefficient and its corresponding dendrogram was constructed using the complete linkage method.

al., 2012). This collection has shown a high rate of phages from urban sewage infecting *P. aeruginosa*. Based on the "kill the winner" theory, it can be suggested that these strains are the most abundant and common in the studied ecosystems. This theory assumes that phages adapt to infect preferentially lineage of bacteria with the highest frequencies in the population (Koskella and Meaden, 2013). Indeed, studies performed in Côte d'Ivoire revealed a high prevalence of these bacteria in urban wastewater (Coulibaly-Kalpy et al., 2016; Guessennd et al., 2013). These latter are directly discharged without prior treatment within the Ebrié Lagoon (Dongo et al., 2013; Scheren et al., 2004). Concerning *S. aureus* infecting phages, we noticed a low rate of isolation, especially from sewage. While, urban sewages and environmental water are known to be a source of phages infecting *P. aeruginosa* (Azizian et al., 2015; Mattila et al., 2015; Weber-Dąbrowska et al., 2016), *S. aureus*' phages isolation seems to be laborious. An evaluation of on-demand isolation of phages revealed that the probability for discovering a phage from a sewage sample against the most common hospital pathogens was 6.1% for *S. aureus* and 79.4% for *P. aeruginosa* (Mattila et al., 2015).

The genetic diversity revealed distinct RAPD profiles showing that the phages were unique and genetically diverse. The RAPD PCR technique allowed us to discriminate between phages lytic against two different bacterial species and genus. As seen in the study of Guriérrez et al. (2011), RAPD PCR is a great approach for typing phages infecting strains belonging to the same species or different species within the same genus or a different genus. This technique was used in previous studies to characterize newly isolated phages against enteropathogenic, enterohemorrhagic, enterotoxigenic, and Shiga-toxin-producing strains of *E. coli* (Dini and De Urraza, 2010; Kakou-Ngazoa et al., 2020). It was also used for typing phages infecting *P. aeruginosa* (Azizian et al., 2015; Kumari et al., 2009; Li et al., 2010).

The dendrogram was deduced using the complete

linkage clustering method. Usually, molecular typing dendrograms in ecology are constructed based on average similarities among objects or on centroids of clusters (Gutiérrez et al., 2011). By contrast, complete linkage clustering demands, for a group to agglomerate with another group, that all objects be related at the given similarity. The result clustering offers a much more robust and contrasting analysis. The clustering obtained with the dendrogram in this study was not surprising because of the phylogenetic relations among phages. Phages isolated with the same species were clustered together. As previously described, phages infecting a specific bacterial host are most similarly and closely related (Hatfull, 2008). This technique was very useful in assessing the diversity of phages isolated in both urban sewage and the Ebrié Lagoon.

Conclusion

Twenty-eight genetically distinct phages lytic against *P. aeruginosa* and *S. aureus* were isolated from water samples in this study. RAPD PCR was useful in performing the comparison between viral strains and did not require earlier genome characterization. These phages and subsequent cocktails could be useful for biocontrol against clinically and industrially relevant bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Q fever in Tunisia, an underestimated infection

Naoufel Kaabia[1*] and Abdelkarim Bahloul[2]

[1]Infectious Diseases Department, Farhat Hached Hospital, Sousse, Tunisia.
[2]Private Infectious Disease's Outpatient Office, Sfax, Tunisia.

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Q fever is a common zoonosis with almost a worldwide distribution caused by *Coxiella burnetii*. Farm animals and pets are the main reservoirs of infection and transmission to humans is usually via inhalation of contaminated aerosols. Infection in humans is often asymptomatic, but it can manifest as an acute disease (usually a self-limited flu-like illness, pneumonia or hepatitis) or as a chronic form (mainly endocarditis). The aim of this review was to describe epidemiological and clinical features of Q fever in Tunisia. A systematic review of all published studies of Q fever in Tunisia was conducted. Although prevalence of immunoglobulins anti-*C. burnetii* was high among animals and blood donors, Q fever was rarely reported and frequently misdiagnosed by physicians.

Key words: Q fever, *Coxiella burnetii*, epidemiology, Tunisia.

INTRODUCTION

Q fever is a zoonotic infection caused by the pathogen *Coxiella burnetii*, which can cause acute or chronic disease with protean manifestations. The designation Q fever (from Query) was made in 1935 following an outbreak of febrile illness in an abattoir in Queensland, Australia. Q fever is a common zoonosis with almost a worldwide distribution. Farm animals and pets are the main reservoirs of infection and transmission to humans is usually via inhalation of contaminated aerosols. Infection in humans is often asymptomatic, but it can manifest as an acute disease (usually a self-limited flu-like illness, pneumonia or hepatitis) or as a chronic form (mainly endocarditis) (Raoult and Marrie, 1995). In Tunisia, researchers started to look for these diseases among patients since 1954 using old technic (Maurin, 1954). In 1984, Kennou and Edlinger confirm existence of antibodies again *C. burnetii* in Tunisian healthy population (Kennou and Edlinger, 1984). Although prevalence of immunoglobulins anti-*C. burnetii* was high among animals and blood donors, Q fever was rarely reported and frequently misdiagnosed by physicians.

The economic impact of this infectious disease is certainly high. First, Q fever is endemic in our livestock and it is a major cause of abortion, in the other hand, in human, Q fever, in its chronic form, can be responsible for long hospital stay and chronic treatment and follow up for some specific patients. Therefore, physicians as well as veterinarians should be aware regarding this zoonosis. This study aims to review epidemiological status and to describe clinical features of Q fever in Tunisia through literature review.

METHODOLOGY

A systematic review was conducted to determine epidemiological

*Corresponding author. E-mail: naoufelkaabia2001@yahoo.fr.

status and clinical features of Q fever in Tunisia. Systematic and comprehensive searches were developed with a clinical librarian and designed for optimal retrieval. The electronic databases MEDLINE/PubMed, Embase, CINAHL and Cochrane Central Register of Controlled Trials (CENTRAL) were searched for literature until July 31, 2021. Key words used were Q fever, *C. burnetii* and Tunisia. All types of studies of any design which describe Q fever among human and animals in Tunisia were searched for. 21 published studies were revised and analyzed and 20 were included in this review (14 in human and 6 in animals), one study was excluded because of duplication. All studies were published in English and/or French languages.

RESULTS AND DISCUSSION

Microbiology

C. burnetii is a short, pleomorphic rod that is a strict intracellular bacterium. While previously designated as a *Rickettsia*, *C. burnetii* has been re-classified as a Proteobacteria, which is closer to *Legionella* and *Francisella* (Stein et al., 1993). In mammals, the usual host cell of *C. burnetii* is the macrophage, which is unable to kill the bacterium. *C. burnetii* lives and multiplies in a single, large, acidic vacuole, which results from the fusion of cell lysosomes (Maurin et al., 1992). In addition, a sporulation-like process protects the organism from the external environment, where it can survive for long periods of time. An important characteristic of *C. burnetii* is its antigenic variation, called "phase variation". When *C. burnetii* express phase I antigen it is highly infectious and a single bacterium is sufficient to infect a human. This is the form that is isolated from animals or humans. After sub culturing *C. burnetii* in cells or embryonated eggs, modification of its lipopolysaccharide (LPS) capsule results in an antigenic shift to the phase II form, which is not infectious. This antigenic shift can be measured and forms the basis for differentiating acute from chronic Q fever (Raoult and Marrie, 1995).

Epidemiology of Q fever in Tunisia

Q fever in animals

Mammals, birds, and arthropods may be reservoirs for *C. burnetii*, but the main reservoir is ticks (Raoult and Marrie, 1995). The most commonly identified sources of human infection are farm animals, such as cattle, goats, and sheep. However, pets, including cats, rabbits, pigeons, hedgehogs, and dogs may serve as sources of urban outbreaks of human disease (Stein and Raoult, 1999; Balti et al., 2021). Q fever can cause abortions in sheep and goats and low birthweight in offspring of cattle. High concentrations of *C. burnetii* may occur in the placenta of infected animals. In Tunisia, a study conducted in 1997, showed that 40% of sheep from different regions were seropositive for *C. burnetii*, in addition, this bacteria was the abortive agent in 17% of sheep and goats (Rekiki et al., 2005). In recent study conducted during 2016-2017, among healthy camels, 44% of them were found to have positive *C burnetii* serology done by ELISA, a meaningful high sero-positivity was observed in female camels with a previous history of abortion (70%) (Selmi et al., 2018). In a study conducted by Selmi et al. (2018) among 327 partially engorged ticks collected from camels, *C. burnetii*, was detected in 3.6%, genotyping and phylogenetic analysis of obtained *C. burnetii* revealed 99 to 100% similarity to the pathogenic *C. burnetii* strains isolated from humans, this finding raises the possibility of the involvement of *Hyalomma* tick species in the active diffusion of these bacteria among camels, other domestic animals and humans (Selmi et al., 2019). Another recent study confirmed the high prevalence of Q fever in animals in Tunisian farms, authors concluded that Q fever increased when the intensive farm was exposed to carnivores and when the cleaning practices were not respected, while it decreased when a suitable quarantine was introduced for any introduction of a new animal. Good hygiene and sanitation practices on-farm should be handled as strategies to deal with this zoonotic pathogen in herds (Barkallah et al., 2018).

Q fever in human

Humans are incidental hosts for infection with *C. burnetii*. Occupational exposure to *C. burnetii* among veterinarians may occur through inhalation of contaminated aerosols arising from the placenta or parturient fluids of infected livestock. *C. burnetii* human infection has also resulted via the following routes: a) trans placental transmission to the fetus, b) intradermal inoculation, and c) blood transfusions. Consumption of raw milk is an additional cause of infection (Salifu et al., 2019; Shishido et al., 2016; Ghanem-Zoubi and Paul, 2020). Q fever can occur in any age group, but infection is most common between the ages of 30 and 70 years. Men are more frequently diagnosed with Q fever than women (Tissot et al., 1992). This can be explained by the fact that men are more exposed to the reservoir, and they have activities that generate aerosols infected by *C. burnetii.* Otherwise, women and children are more likely to have asymptomatic infection (Tissot et al., 1992). Since the clinical presentation is nonspecific, recognition of cases depends upon astute clinicians and the availability of a reference laboratory. Thus, incidence figures for the disease vary widely. Q fever is rampant in endemic mode with the possibility of epidemic outbreak.

Blood samples (1888) from febrile and non-febrile patients from six African countries were investigated retrospectively for Q fever infection by molecular assays showed a prevalence ranging from 0.3 to 0.5% (Angelakis et al., 2014). In Tunisia, Q fever is an endemic

disease, but rarely diagnosed and confirmed by physicians. In fact, in 1983, Kennou and Edlinger (1984) found that prevalence of *C. burnetii* antibodies among healthy population was 8.7% (3). Another study conducted in 1993, noted that 26% of blood donors in the Tunisia eastern central region (Sousse, Monastir) had antibodies to *C. burnetii*, majority of them were male (Letaief et al., 1995). During the same period, among 300 inpatients presented with acute fever, *C. burnetii* serology was done for all patients, showed a typical profile of acute Q fever among 2% of patients and old contact with *C. burnetii* in 29% of cases (Omezzine-Letaief et al., 1997). In addition, a serological survey carried out during 2004, among 47 inpatients with unexplained fever, acute Q fever had been confirmed in 8 cases (17%) (Kaabia et al., 2006).

Clinical manifestations

Patients with Q fever present with a wide spectrum of disease manifestations. While for some patients the clinical manifestations of acute or chronic infection are severe, the clinical signs and symptoms of Q fever are mild or absent in others.

Acute Q fever (AQF)

The incubation period for acute infection is approximately 20 days (range 14 to 39) (Raoult et al., 2005). Patients with acute Q fever can present with any of the following manifestations.

Flu-like illness: A self-limited flu-like syndrome is the most common manifestation of acute infection. The onset is typically abrupt, with high-grade fevers (40°C), fatigue, headache, and myalgia being the most frequent associated symptoms. In such patients, the headaches are usually severe and can be associated with photophobia. Febrile episodes usually last from one to three weeks.

Pneumonia: Most cases of Q fever pneumonia are mild, and patients present with a non-productive cough and fever. However, acute respiratory distress may occur in some patients. Findings on the chest radiograph are not specific and resemble a viral pneumonia. Pleural effusion may also occur, but it is uncommon. In addition to respiratory symptoms, patients often have extra pulmonary manifestations including severe headaches, myalgia, and arthralgia. Symptoms can last from 10 to 90 days. Mortality rates are low (ranging 0.5 to 1.5%) (Raoult and Marrie, 1995). Among 240 Tunisian patients admitted for acute exacerbations of chronic obstructive pulmonary disease, acute Q fever was diagnosed in 6.6% (Messous et al., 2018).

Hepatitis: Patients with hepatic involvement present with high liver enzymes and can also have: hepatomegaly, which usually occurs without jaundice, an acute febrile episode. Prolonged fever of unknown origin with characteristic granulomas on liver biopsy. The granulomas appear to be "doughnut-like" because they contain a lipidic vacuole surrounded by a fibrinoid ring (Dauby et al., 2020).

Other manifestations of acute Q fever: They include maculopapular or purpuric rash (10%), pericarditis and/or myocarditis (1%), myocarditis can be a particularly severe (Fournier et al., 2001), aseptic meningitis and/or encephalitis (1%) (Bernit et al. 2002). Other rare features were described like neuritis, hemolytic anemia, thyroiditis, gastroenteritis, pancreatitis, lymphadenopathy mimicking lymphoma, erythema nodosum, orchitis, and acute acalculous cholecystitis (Rolain et al., 2003).

In Tunisian studies, the main clinical presentations of acute Q fever were interstitial pneumonia, isolated fever or associated with cytolysis (Bellazreg et al., 2009; Kaabia and Letaief, 2009), and one case of chronic fever of unknown origin with granulomatous in liver biopsy (Omezzine-Letaief et al., 1997). Recently two cases of acute Q fever were published, the first case, was a 19 year-old healthy patient with myocarditis (Hammami et al., 2021), the second, an acute fever in patient treated by Etanercept for Ankylosing Spondyloarthritis (Guiga et al., 2021), both patient treated by doxycycline and their outcome was favorable.

Q fever in pregnancy

Pregnant women presenting with acute Q fever are significantly more likely to be asymptomatic than other patients, however, Q fever may result in obstetrical complications such as spontaneous abortion, intrauterine growth retardation, intrauterine fetal death, oligoamnios, and premature delivery (Ghanem-Zoubi and Paul, 2020), in Tunisian pregnant woman, Q fever studies are rare, its prevalence is unknown, only one study investigated the relationship between miscarriage in humans and infections caused by zoonotic bacteria and genital pathogens, did not find any link between *C. burnetii* and abortion (Smaoui et al., 2019).

Chronic Q fever (CQF)

It is rare; reported in less than 5% after acute illness, occurred within few months, years or decades after AQF. Pregnancy, cardiac and valve diseases and immunosuppression are high risks for later CQF.

Infective endocarditis with negative blood cultures on prosthetic valve or preexisting lesions is the most common form of CQF (Kampschreur et al., 2015), followed by

infection on aneurysm or vascular prosthesis (Botelho-Nevers et al., 2007). The prognosis of CQF is pejorative with high mortality.

In a recent systematic review of *C. burnetii* epidemiology in Africa, *C. burnetii* accounted for 1 to 3% of infective endocarditis in Tunisia (Ben et al., 2014). Rekik et al. (2009) in their series of 48 patients with prosthesis valve endocarditis, collected during 10 years (1997-2006) from tertiary care hospital in central Tunisia, one patient had chronic Q fever. Others cases reports were published; all were endocarditis with negative blood culture in immunocompetent patient. The first case, a 35-year-old man, who had recurrent endocarditis (4 episodes) on mitral prosthetic valve. Q fever serology requested only in the fourth episode, showed profile of chronic Q fever. Despite appropriate antibiotics, the patient died after prosthetic valve disinsertion (Ameur et al., 1997). The second case, a 42-year-old patient, with history of mitral and aortic rheumatic disease, presented shortness of breath and fever, since more 3 months, he had splenomegaly. Cardiac echocardiography showed abscess of the mitro-aortic trigone. Blood culture was sterile*, C. burnetii* serology confirms the diagnosis of chronic Q fever with high titer of antibodies phase I. Patient underwent mitral and aortic valves replacement. He received doxycycline, hydroxychloroquine and ofloxacine for 18 months, with favorable outcome (Fradi et al., 2006). The third case, 48-year-old male patient, presented native aortic valve endocarditis with negative blood culture. Biological prosthesis insertion was done because of hemodynamic instability due to acute heart failure. Unfortunately, patient experienced postoperative bleeding with disseminated intravascular coagulation and died few days after cardiac surgery. Serology for *C. burnetii* was positive, with high titer for IgG phase I (1/6400). Culture of the valve remained sterile. Quantitative real-time PCR (qPCR) analysis of the valvular sample was strongly positive (355, 395, and 350 copies/mL) for *C. burnetii*. Whole genome sequencing of the strain was performed directly on the valvular sample to test whether direct sequencing would be feasible for such highly positive sample and to provide genomic data on a Tunisian strain (Delaloye et al., 2017)

Diagnosis

Nonspecific laboratory findings

In AQF, leucocytes count is often normal whereas thrombocytopenia is found in 25 to 30% of cases. Increased liver enzymes could be found up to 71% of cases, cholestasis is less common. In Tunisian series cases, normal WBC or leucopenia, and high liver enzymes (ALT/AST) were the most frequent laboratory findings (71.5%), followed by thrombocytopenia noted in57% (Bellazreg et al., 2009).

In CQF, an increase inflammatory markers is frequently seen sometimes along with circulating immune complexes, rheumatoid factors, microscopic hematuria and cryoglobulinemia.

Confirmatory diagnostic tests

Serologic Testing (Immunofluorescence Assay): The diagnosis of Q fever is confirmed serologically in majority of cases. In AQF, a fourfold rise in titer of phase II IgG between acute and convalescence samples is diagnostic of Q fever. A single serum specimen could be used for the diagnosis of AQF, a titer of phase II IgG > 1/128 in patient with prolonged febrile illness and clinical manifestations of Q fever is suggestive of AQF. IgM antibodies have limited diagnosis value. In CQF, continued increasing phase I IgG antibodies with a titer ≥ 1/1024 along with identifiable site of chronic infection such as endocarditis or endovascular infection is diagnostic of CQF (Kampschreur et al., 2015).

Nucleic acid detection by PCR: PCR has been successfully employed to detect DNA in both cell cultures and clinical samples (Stein et al., 1993). PCR testing can be performed on excised heart valve tissue from the site of active infection (even if frozen or embedded in paraffin), serum, cerebrospinal fluid, pleural fluid, bone marrow, bone and liver biopsies, breast milk, placenta, and fetal tissue**.** PCR testing was used to diagnose patients whom were suspected of having acute infection but initial serologic testing reveals no or low levels of antibodies. The PCR generally remains positive for 7 to 10 days in acute infection. PCR testing is also helpful in confirming the serologic diagnosis of endocarditis or vascular infection in patients who have persistent elevations of IgG anti-phase I titers.

Culture

Although *C. burnetii* does not grow in routine blood cultures, culture of this organism can be performed on blood, bone biopsies, cardiac valves, and vascular samples.

Isolation of the bacteria by culture is not recommended for routine diagnosis. It is difficult, time consuming and dangerous requiring a biosafety level 3 laboratory.

In Tunisia, few referent laboratories, from academic hospitals, perform Q fever serology, they look for total Immunoglobulins against *C burnetii,* without differentiating between Phase I and II.

Treatment

AQF

Majority of AQF resolve spontaneously within 2 to 3

weeks. However, symptomatic or suspected AQF should be treated whereas the treatment is not routinely recommended for asymptomatic form or resolved symptoms if there is no risk factors to develop CQF. The first line antibiotic is doxycycline with a duration of 2 weeks. If this antibiotic is contraindicated, fluoroquinolones, macrolides, rifampicin and trimethoprim/sulfamethoxazol (cotrimoxazol) could be used. Thorough clinical assessment for all patients with AQF is recommended to look for any immunosuppression, pregnancy and cardiac valve defect or valve disease.

Clinical and serologic follow up monitoring is mandatory for all patients after AQF. This monitoring would be closer and longer in patients with cardiovascular risk factors (Melenotte et al., 2020).

CQF

The presence of a nidus of infection with increase of phase I IgG titer (≥ 1/1024) is an indicator to start treatment. The regimen of choice is the combination of Doxycycline with Hydroxychloroquine. The duration of the treatment is 18 to 24 months in case of infective endocarditis or vascular infection. For other CQF sites the duration depends on the clinical and serologic response. Surgical treatment might be necessary if no response to antibiotics. The hydroxychloroquine is contra-indicated in case of G6PD deficiency and in case of retinal or visual field deficits. In such situations, fluoroquinolones, rifampicin, macrolides and cotrimoxazol could be an alternative for the treatment.

Cured case is defined by decrease of phase I IgG (≤1/200) with recovery of clinical symptoms. After the treatment serologic monitoring every 6 months should be done for at least 5 years, lifelong if sever valvular disease (Melenotte et al., 2020).

Pregnant woman

Treatment of AQF in pregnant woman reduces risks of adverse consequences for the fetus, and conversion to CQF. Cotrimoxazol is the antibiotic of choice; it should be given throughout pregnancy except the last two weeks. Macrolides would be alternative for treatment of CQF in pregnant women (Ghanem-Zoubi and Paul, 2020).

Perspective

Actually, based on the recent knowledge on Q fever there is tendency to change the old classification of acute and chronic Q fever to: a) acute infection including flu like illness, pneumonia, hepatitis, acute endocarditis, and other rare manifestation, and b) persistent localized infection, including chronic endocarditis, vascular infection, bone and joint infection and other forms of persistent infection. Many retrospective studies highlighted the role of anticardiolipin antibodies as a useful biological predictive marker for acute Q fever complications, and relationship between persistent *C. burnetii* infection and non-Hodgkin lymphoma (Melenotte et al., 2020).

Conclusion

In Tunisia, although acute Q fever is endemic among animals and human, only few chronic Q fever cases were published, this disease is not considered as communicable diseases for notification that is why there is no available data from the official health authorities. The real frequency of *C. burnetii*, was not known, especially among patients with negative blood culture endocarditis. We think that chronic Q fever is underestimated in our country for many reasons, mainly lack of awerness among physicians regarding this infection, and unavailability of Q fever serology in majority of our laboratories. More epidemiologic studies are needed to determine the accurate incidence and prevalence of this zoonosis in Tunisia, especially among patient with negative blood culture endocarditis and women with recurrent miscarriage.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Prevalence of SEA and SEB producing *Staphylococcus aureus* isolated from foodborne- outbreaks in Iran

Mehrnaz Mirzababaei[1], Mohammad Mehdi Soltan Dallal[1,2*], Ramin Mazaheri Nezhad Fard[1,2], Hossein Masoumi Asl[3] and Mahdieh Pourmoradian[1]

[1]Division of Food Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
[2]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.
[3]Center for Communicable Disease Control, Ministry of Health and Medical Education, Tehran, Iran.

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***Staphylococcus aureus* is an important pathogen causing a wide variety of diseases such as skin infections, food poisoning, pneumonia and septicemia in humans and animals. Among the bacterial agents of food poisoning, S. aureus is the most common agent worldwide. The aim of this study was to assess prevalence of SEA, SEB enterotoxin producing S. aureus isolated from foodborne outbreaks in Iran. In this study, 313 diarrheal samples from 120 outbreaks were collected from December 2017 to August 2018. Isolates were identified using classical methods. The antimicrobial resistance patterns of the S. aureus isolates were assessed using standard disk-diffusion and E-test methods. Presence of sea and seb genes was investigated using polymerase chain reaction (PCR). Data were analyzed using SPSS and Excel Software as well as statistical tests. In this study, 55 samples (17.6%) from an overall number of 313 samples from food outbreaks were identified as S. aureus, which were assessed for their antimicrobial susceptibility. The highest contamination belonged to Yazd (50.9%) and Semnan (29.1%) Provinces. However, no contaminations of S. aureus were seen in Zanjan Province. The S. aureus was more common in females (50.9%) than males. Furthermore, S. aureus isolates were mostly resistant to penicillin (81.8%) and completely susceptibile to vancomycin. Of 55 isolates of S. aureus, four isolates (7.3%) were positive for sea and one (1.8%) for seb genes. The current study has shown that S. aureus food infection is one of the most common foodborne diseases, caused by the ingestion of staphylococcal enterotoxins produced by enterotoxigenic strains of staphylococci. Therefore, further screening and monitoring programs are suggested for the prevention of staphylococcal infections.**

Key words: *Staphylococcus aureus*, foodborne outbreaks, antibiotic resistance, *sea*, *seb.*

INTRODUCTION

Foodborne infection is one of the most widespread public health problems worldwide, which can be caused by the consumption of foods contaminated with microorganisms or chemicals (World Health Organization (WHO), 2015). The WHO has described foodborne outbreak as “a foodborne outbreak happens when two or more people

*Corresponding author. E-mail: msoltandallal@gmail.com. Tel: +98-21-: 88992971.

Table 1. List of primers used in PCR of *sea* and *seb* genes.

Primer	Sequence (5´–3´)	bp*	Ref.
seb-F	TTTTTCTTTGTCGTAAGATAA	477	15
seb-R	CCAACGTTTTAGCAGAGAAG		
sea-F	TAAGGAGGTGGTGCCTATGG	127	15
sea-R	CATCGAAACCAGCCAAAGTT		

*bp, amplicon size; ref., references.

show the same symptoms of an illness after consuming a common food or drink source" (World Health Organization (WHO), 2008; Kirk et al., 2015). *Staphylococcus aureus* is one of the most important microorganisms causing foodborne outbreaks (Masoumi et al., 2015). This bacteria is a Gram-positive aerobic bacteria discovered in the normal flora of various parts of the human and animal bodies (Lowy, 1998). In fact, *S. aureus* is an opportunistic pathogen that can induce food poisoning by producing enterotoxins (Hennekinne et al., 2012). Staphylococcal enterotoxins (SEs) are members of a family of more than 20 different staphylococcal and streptococcal exotoxins (Salgado-Pabon et al., 2014). These toxins share a common phylogenetic relationship, structure, function, and sequence homology (Soltan Dallal et al., 2016). Staphylococcal enterotoxins A and B (SEA and SEB) are the most significant enterotoxins produced by *S. aureus* (Pinchuk et al., 2010). SEA is often associated to foodborne outbreaks by *S. aureus* and SEB causes food poisoning (Rusnak et al., 2004). Many foods can be contaminated with SEs, particularly foods that contain carbohydrates and proteins (such as meats, eggs and dairy products). Most of the SE food poisoning cases are due to direct contact of hands with foods (Soltan Dallal et al., 2010). Production of SEs develops at optimum temperature (20-37°C) and pH (4–4.7). The most common symptoms of SE food poisoning are nausea, diarrhea, vomiting and abdominal cramps, which usually happen within 2-6 h after eating contaminated foods (Fletcher et al., 2015). Little is known about the implication of SEA/SEB-producing *S. aureus* in foodborne outbreaks in Iran. Therefore, the aim of this study was to determine the prevalence of SEA/SEB-producing *S. aureus* isolates from foodborne outbreaks in five Iranian provinces and to study the antimicrobial susceptibility patterns of these isolates.

MATERIALS AND METHODS

Sample collection

In this study, human diarrheal samples of foodborne outbreaks were collected from five Iranian Provinces of Tehran, Semnan, Kurdistan, Hamadan and Yazd from December 2017 to August 2018. The study was previously approved by the Ethical Committee of Tehran University of Medical Sciences (Code No. IR.TUMS.VCR.REC.1397.836). These samples were systematically sent to Accredited Food Microbiology Laboratory of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, by local health centers for microbiological identification. Results were reported to the centers confidentially. All activities within the laboratory were officially approved by the administrators.

Isolation and identification of *S. aureus*

Stool samples and rectal specimens were inoculated into Chapman culture media (Merck, Germany) and incubated at 37°C for 24 h. Routine biochemical tests of Gram staining, catalase, maltose fermentation, VP, DNase, coagulase and sensitivity to novobiocin and polymyxin B were carried out for the yellow colonies of *S. aureus* (Koneman et al., 2008).

Antimicrobial susceptibility test (AST)

The AST was carried out for the isolates that were phenotypically identified as *S. aureus* using disk diffusion and minimum inhibitory concentration (MIC). Disc diffusion test was carried out using Kirby-Bauer method. Bacterial suspensions of 0.5 McFarland turbidity provided were cultured on Muller-Hinton agar (MHA) (Merck, Germany). Routine clinically used antibiotic discs of penicillin (10 U), vancomycin (30 µg) and oxacillin (1 µg) (Merck, Germany) were used for the test. Results were reported based on the CLSI guidelines (Patra et al., 2011). The MIC test for vancomycin was carried out using E-tests. Bacterial suspensions of 0.5 McFarland turbidity were prepared from overnight cultures of *S. aureus* isolates on plate count agar (PCA) media. Suspensions were cultured on MHA plates. After a few minutes, vancomycin stripes (Liofilchem, MTS) were set on MHA plates and incubated at 37°C for 24 h. Results were reported based on the CLSI guidelines (Patra et al., 2011).

Detection of *sea* and *seb* genes using polymerase chain reaction (PCR)

The *sea*, and *seb* genes were detected using PCR and specific primer pairs (Table 1). Briefly, DNA extraction was carried out using alkaline lysis method (Zouharova and Rysanek, 2008). The PCR reactions included 12.5 µl of Master Mix (Genfanavaran, Iran), 10.1 µl of distilled water, 0.5 µl (10 pM) of each primer (Genfanavaran, Iran), 50 ng of DNA template and 1 U of Taq DNA polymerase (Genfanavaran, Iran). The thermal cycling used for the PCR was as follows: initial denaturation at 95°C for 5 min; then, 35 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 90 s. The final extension was carried out at 72°C for 5 min. The *S. aureus* SINA strains were used as positive and DNA-free reactions as negative controls.

Statistical analysis

Data were statistically analyzed using SPSS Software v.18 (IBM

Analytics, USA), chi-square and Fisher exact tests with 95% confidence. A P-value of 0.05 was recognized as significant.

RESULTS

Prevalence of *S. aureus*

From December 2017 to August 2018, 313 diarrheas samples were collected from 120 foodborne outbreaks. In general, 55% of these isolates (17.6%) were identified as *S. aureus*. As shown in Table 2, *S. aureus* was mostly isolated from Yazd (28 isolates, 50.9%) and Semnan (16 isolates, 29.1%) Provinces. Prevalence of *S. aureus* was high in a patient age range of 20-29 years (17 patients, 30.9%); mostly from females (28 isolates, 50.9%). Most *S. aureus* were isolated in January and July (nine isolates each) with no strains isolated in March. Most isolates of *S. aureus* belonged to salad and rice-stew; however, food sources were unclear in most outbreaks. None of these results were statistically significant ($P > 0.05$). The most common infection symptom was watery diarrhea in 55 cases (100%); however, no dysentery symptom was reported. Diarrhea, nausea and fever symptoms showed a significant association with the isolation of *S. aureus* from foodborne outbreak samples ($P < 0.05$).

Antimicrobial susceptibility

The highest antibiotic resistance in *S. aureus* belonged to penicillin (45 isolates, 81.8%), while all isolates were susceptible to vancomycin (55 isolates, 100%) (Figure 1). Furthermore, nearly eight of the isolates (14.5%) were resistant to oxacillin.

Prevalence of *sea* and *seb* genes

The *sea* and *seb* genes were detected in four (7.3%) and one (1.8%) isolates, respectively (Figures 2 and 3). All detected *sea* genes belonged to isolates from Yazd Province.

DISCUSSION

S. aureus is a commensal and opportunistic pathogen that cause a wide range of infections, including superficial invasive infections (Lowy, 1998). This bacterium is the major pathogen of nosocomial and community-acquired infections (Chaibenjawong and Foster, 2011; Soltan Dallal et al., 2016). The current study was carried out on 313 foodborne diarrheal samples to isolate *S. aureus*. Fifty-five isolates (17.6%) were identified as *S. aureus*. In a study in Brazil, 2926 foodborne outbreaks were included (de Oliveira et al., 2018). The most common bacterial causes of the foodborne outbreaks were *Salmonella* (30%) and *S. aureus* (23.3%). Rates of *S. aureus* isolation mainly vary within various countries. Differences in hygiene, population and diet can result in differences in causative agent prevalence of the foodborne outbreaks. Unlike other bacteria, if a person is contaminated by *S. aureus* in the nose or skin, food contamination control protocols cannot be possible just by washing hands with soap and water while preparing and processing foods. Therefore, the infection control of *S. aureus* needs more precise monitoring protocols (de Oliveira et al., 2018; Gould et al., 2013; Kozak et al., 2013). In the current study, most of the *S. aureus* isolates were collected from 28 samples (50.9%) from Yazd and 16 samples (29.1%) from Semnan Provinces. In a study by Madahi et al. (2013) in Isfahan, the infection rate of chicken nuggets with *S. aureus* was 5.7% (Madahi et al., 2015). In another study by Feizi et al., the rate of *S. aureus* infection in chicken meats was 3.5% (23). As previously highlighted, the infection rate of *S. aureus* depends on hygiene level, weather condition, population, diet type and food source (Soltan Dallal et al., 2015). In this study, the highest age group with *S. aureus* infections was 20–29 year-old group (17 isolates, 30.9%). Furthermore, rates of infections in females and men included 50.9 and 49.1%, respectively. In a study in the United States, the predominant age group was reported as 40-49 year-old group (48%)(Bennett et al., 2013). Moreover, females were mostly infected by *S. aureus* (52%); similar to the present study. In a study in Japan, the most frequent age group infected with *S. aureus* foodborne outbreaks was less than ten years, mostly including girls (55.3%)(Asao et al., 2003). Based on the findings from the current study and similar studies, age groups of the patients infected with *S. aureus* differ in geographic regions.

In this study, the most common infection symptom was watery diarrhea in 55 cases (100%), followed by vomiting in 49 (89.1%), nausea in 49 (89.1%), abdominal cramp in 34 (61.8%), fever in three (5.5%) and headache in 22 (40%) cases. In other studies, similar symptoms were reported from the patients (Soltan Dallal et al., 2015; Guidi et al., 2018; Teague et al., 2013). In these studies, diarrhea has been shown as the most prevalent symptom of *S. aureus* infections. Normally, the *S. aureus* food toxicity is due to the production of bacterial enterotoxins. Staphylococcal enterotoxin contaminated foods have been shown to cause sudden vomiting, which may be followed by diarrhea after 4-6 h. Classic symptoms of the infection include nausea, vomiting, abdominal pain and diarrhea, while the headache is less common and fever occurs in only 25% of the patients (Landgraf and Destro, 2013). In the present study, the highest infection rate of *S. aureus* was seen in January and July (9 and 16.4%, respectively). In a study since 2006 to 2011 by Masoumi et al. (2015) foodborne outbreaks were studied; of which, the highest number of outbreaks occurred in August and September (17.8 and 13.6%, respectively) (Masoumi et al., 2015). In a study by Soltan Dallal et al. (2016) the

Table 2. Analysis of *S. aureus* isolated from foodborne outbreaks.

Prevalence	*S. aureus* (%)	*P*-value
Province		
Tehran	1 (1.8)	
Semnan	16 (29.1)	0.118
Kurdistan	5 (9.1)	
Hamadan	5 (9.1)	
Yazd	28 (50.9)	
Age group		
< 10	15 (27.3)	
10–19	13 (23.6)	
20–29	17 (30.9)	0.69
30–39	4 (7.3)	
40–49	4 (7.3)	
50 ≥	2 (3.6)	
Gender		
Female	28 (50.9)	0.42
Male	27 (49.1)	
Month		
January	9 (16.4)	
February	5 (9.1)	
March	4 (7.3)	
April	6 (10.9)	
May	1 (1.8)	
June	1 (1.8)	0.42
July	9 (16.4)	
August	8 (14.5)	
September	7 (12.7)	
October	3 (5.5)	
November	2 (3.6)	
December	0 (0)	
Clinical symptom		
Watery diarrhea	55 (100)	0.0001
Dysentery	0 (0)	0.05
Vomiting	49 (89.1)	0.045
Nausea	49 (89.1)	0.045
Abdominal cramp	34 (61.8)	0.097
Fever	3 (5.5)	0.0001
Headache	22 (40)	0.76
Food type		
Junk food	1 (1.8)	
Salad	5 (9.1)	
Spaghetti	4 (7.3)	
Rice-stew	5 (9.1)	
Chelo kebab	3 (5.5)	
Mushroom	2 (3.6)	
Falafel	3 (5.5)	
Omelet	4 (7.3)	

Table 2. Cont'd.

Chicken	5 (9.1)
Dairy	2 (3.6)
Water	1 (1.8)
Unknown	20 (36.4)

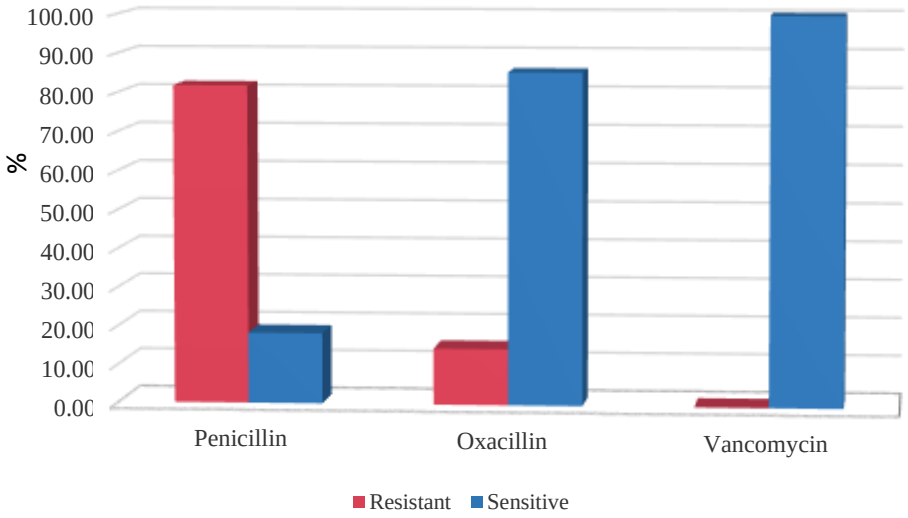

Figure 1. Antibiotic susceptibility of *S. aureus* isolated from foodborne outbreaks.

highest number of outbreaks was reported in September (30.13%) (Soltan Dallal et al., 2016). Studies have shown that the foodborne outbreaks mostly occur in warm months of the year; similar to that the current study has. In this study, the most common food sources for the outbreaks of *S. aureus* included salad, chicken and rice-stew for 15 samples (27.3%). In a study by Mossong et al., (2015) pasta salad (82%) was more likely to be contaminated with *S. aureus* (Mossong et al., 2015). Salad is one of the foods that need to be handled directly and can be easily contaminated by the carriers of *S. aureus*. A study in Germany showed that a food outbreak that caused infections in seven people was due to the consumption of ice cream contaminated with *S. aureus* (Fetsch et al., 2014). In the present study, two isolates (3.6%) of dairy foods were contaminated with *S. aureus*. Due to the uncertainty of contamination sources, it cannot be stated definitely that dairy products such as ice cream cause contaminations in a large number of cases in Iran. Ice cream is a high-risk food for *S. aureus* infections. Milk in ice creams is heated using a low temperature, allowing survival of the naturally occurring infectious bacteria. Other risk factors include additives in ice creams (Fetsch et al., 2014). However, the infection was mostly detected in salads, rice-stews and chickens in the current study.

As drug-resistant *Staphylococcus* spp. cause nosocomial and other communicable infections, these bacteria are considered one of the major threats to public health (Sarrafzadeh et al., 2014; Haran et al., 2012; Ateba et al., 2010). In the present study, the highest rate of resistance was observed toward penicillin (81.8%). Penicillin is widely used to treat infections in humans and animals. However, overuse of this antibiotic has led bacterial resistance. Unnecessary administration of antibiotics for therapeutic purposes and inappropriate and unscientific use of antibiotics are other factors that cause selection of antibiotic-resistant bacteria (Yavari, 2013). Antibiotic resistance restricts cure infection ability; therefore, strict rules must be used to prevent the spread of drug resistance. Antimicrobial drugs are used for the treatment, prevention and control of infectious diseases as well as dietary complements in foods. Relationships between the use of antimicrobial drugs in livestock and the selection of resistant bacteria in food chains have been debated widely (Yavari, 2013). In a study by Al-Bahry et al. (2014) in Oman, most of *S. aureus* isolates from food samples were multidrug resistant. The highest antibiotic resistance rate was observed for ampicillin and penicillin (86.3 and 85.93%, respectively) while vancomycin resistance was seen in a few isolates of *S. aureus* (5%) (Al-Bahry et al., 2014). Acco et al. (2003) showed that 70% of the *S. aureus* isolated from individuals involved in food baking were resistant to

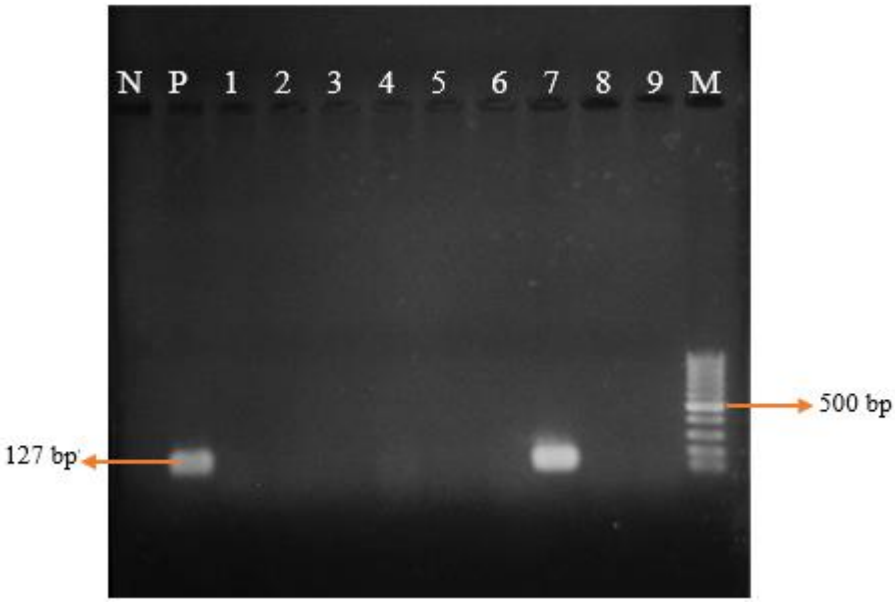

Figure 2. Agarose gel electrophoresis of *sea* gene amplicons of *S. aureus* isolated from foodborne outbreaks. Lane M, DNA ladder (100 bp); Lane N, negative control; Lane P, positive control (*S. aureus* strain SINA); and Lane 7, *sea*-positive *S. aureus* isolate from foodborne outbreaks.

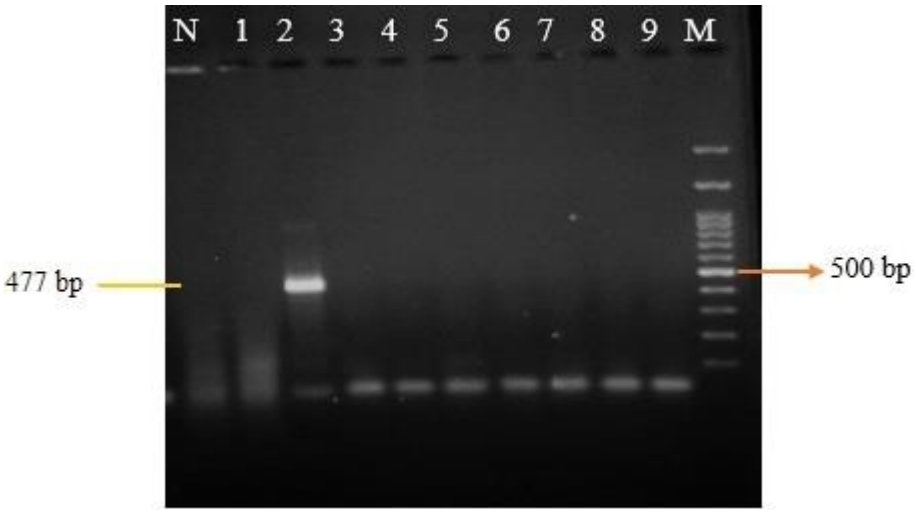

Figure 3. Agarose gel electrophoresis of *seb* genes of *S. aureus* isolated from foodborne outbreaks. Lane M, DNA ladder (100 bp); Lane N, negative control; and Lane 2, *seb*-postive *S. aureus* isolate from foodborne outbreaks

penicillin (Acco et al., 2003). In several countries, large-scale studies have detected penicillin resistance in approximately 60% of studied *S. aureus* isolates (Zarkesh et al., 2016). In this study, a high resistance rate to penicillin was observed; similar to other studies.

One of the most important virulence determinants of *S. aureus* are enterotoxins (SE). Over 15 types of SE with similar protein structures have been characterized (Pourmand et al., 2009). These toxins are not digested by the intestinal proteases such as pepsin and are highly resistant to high temperatures. For example, SEA preserves its biological properties after 28 min at 121°C (Nazari et al., 2014). Due to its thermo-resistance trait, SEA must be avoided in foods. As the taste and color of foods are not changed by SEA, usual cooking methods do not eliminate this toxin. In most parts of the world,

nearly 50% of foodborne diseases are caused by the SEA. The enterotoxins in raw meats should be assessed before eating since these toxins are poisonous even at very low levels (0.5 ng/ml) (Alizadeh and Amini, 2015). Enterotoxins of *Staphylococcus* spp. can be identified using immunological methods (Pourmand et al., 2009); however, use of these methods is usually limited. Therefore, use of DNA-based methods has become further popular. In this study, *sea* and *seb* enterotoxin genes were detected in four (7.3%) and one (1.8%) *S. aureus* isolates, respectively. In a study by Johler et al. (2015) 14 patients with *S. aureus* intoxication were studied. The prevalence of *sea* enterotoxin gene was higher, compared to that of other enterotoxins (Johler et al., 2015). In Asgarpoor et al. (2018) study on 136 samples of *S. aureus* infected individuals, prevalence of *sea* and *seb* genes was 23.9 and 13%, respectively (Asgarpoor et al., 2018). Gholamzad et al. (2015) investigated 80 food samples suspected to *S. aureus* contamination and showed presence of the *seb* gene in 54 samples (Gholamzad et al., 2015). The *sea* gene is the most common gene within toxin-producing isolates. Moreover, all enterotoxins have similarities in gene structures and sequences; mostly encoded by mobile genetic elements (MGEs) such as bacteriophages, plasmids, transposons, insertion sequences (IS), integrons and pathogenic islands (PI)(Baba et al., 2002).

Conclusion

In this study, prevalence of *S. aureus* foodborne outbreaks in various regions of Iran was investigated for the first time. Overall, the current study showed that *S. aureus* accounts for a high rate of food contaminations. The *sea* and *seb* genes encoding SE were detected in eight *S. aureus* isolates; hence, elimination of the bacteria in foods is necessary. Furthermore, increased bacterial food poisoning and resistance to penicillin and oxacillin have raised public concerns. Despite reports on vancomycin resistance in *S. aureus* isolates, all our *S. aureus* strains were susceptible to this antibiotic.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Ethics committee

The study was previously approved by the Ethical Committee of Tehran University of Medical Sciences (Code No. IR.TUMS.VCR.REC.1397.836).

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