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

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Review

Listeria monocytogenes in food: Control by monitoring the food processing environment

Dara Leong[1], Avelino Alvarez-Ordóñez[1], Piet Jooste[2] and Kieran Jordan[1]*

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***Listeria monocytogenes* is a foodborne pathogen that is widely dispersed in the environment; it is found in soil, water and plant material, and can grow at refrigeration temperature and at unfavourable conditions of pH (up to pH 4.7) and salt (up to 10%). It can persist in the harsh conditions of the food processing environment from which it can contaminate food. Listeriosis, infection with *L. monocytogenes*, can be mild but the ability of the pathogen to cross the epithelial barrier of the intestinal tract, the blood brain barrier and the feto-placental barrier can also result in more severe illness including bacteremia and meningitis or spontaneous miscarriage. Although relatively rare, infection with *L. monocytogenes* can have a mortality rate of up to 30%, resulting in a serious hazard, particularly for the high risk groups of the elderly and immunocompromised individuals. As consumer demand for less processed, less preserved, longer shelf-life ready-to-eat food increases, the threat of *L. monocytogenes* to public health and the food industry continues to rise. In addition to being a public health threat, *L. monocytogenes* is a major economic burden on industry in terms of costs of analysis and potential product recalls. Awareness of its ubiquitous nature and understanding of its physiology and survival are important aspects of its control in the food processing environment with the aim of reducing the public health concern. Appropriate methodologies are required for its detection and isolation. Characterisation of strains by pulsed field gel electrophoresis (PFGE) and other genotypic methods can facilitate identification of putative contamination routes. Whole genome sequencing (WGS) of outbreak strains is becoming a part of outbreak investigation. Such WGS will lead to a greater understanding of the physiology of the organism as well as contribute to understanding epidemiology and pathogenicity. However, despite the advances, the best mechanism of public health protection is still prevention. Awareness of its presence, and control by conventional hygiene methods or by novel biocontrol methods such as bacteriocins and bacteriophage will help prevent cross-contamination of food from the environment and therefore reduce the public health burden.**

Key words: *Listeria monocytogenes*, food, occurrence, prevalence, control.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that causes the disease listeriosis. Although rare, the mortality rate of listeriosis is 25% worldwide (de Noordhout et al., 2014) and with a hospitalisation rate of >95% (Scallan et al., 2011), it ranks as the third most serious foodborne disease. Schlech (2000) has reviewed the clinical

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Table 1. Major outbreaks of foodborne listeriosis since 2010.

Year	Place	No. of cases (deaths)	Food type	Serovar	References
2009/2010	Austria/Germany Czech Republic	34 (8)	Quargel	1/2a (2 strains)	Fretz et al., 2010
2011-2012	US	146 (31)	Cantaloupe	Multiple strains of 1/2a and 1/2b	CDC, 2011
2012	14 US states	20 (4)	Ricotta salata cheese		CDC, 2012
2012	Spain	2	Fresh cheese	1/2a	de Castro et al., 2012
2013	5 US states	6 (1)	Farmstead cheeses		CDC, 2013
2014	California and Maryland (USA)	8 (1)	Dairy products		CDC, 2014b
2013-2014	Denmark	41 (17)	Spiced lamb roll, pork, sausages, liver pâté and other meat products		Anonymous 2015a.
2014-January 2015	12 US states	35 (7)	Caramel apple	4b	CDC 2015a
2015	4 US states	10 (3)	Ice cream		CDC 2015b

manifestations of listeriosis, and there have been many recent high-profile outbreaks of listeriosis worldwide that have resulted in numerous fatalities (Table 1). In South Africa, Fredericks et al. (2015) reviewed a particular issue of listeriosis-associated brain stem encephalitis (Fredericks et al., 2010).

Among the many species of the genus *Listeria*, *L. monocytogenes* is the only one that causes disease in humans, apart from a few reported cases of disease caused by *Listeria ivanovii* (Guillet et al., 2010), although *L. ivanovii* can be pathogenic for animals. None of the other species of the genus have been reported to cause disease.

In addition to being a public health risk, *L. monocytogenes* is an economic burden on the ready-to-eat (RTE) food industry. Ready-to-eat foods are the most vulnerable to *L. monocytogenes* as they do not have a heating or other antibacterial step between production and consumption. The economic burden includes the cost of analysis of samples, the costs, both financial and reputational, of recall of a contaminated product and the possible litigation costs, if the food is shown to have caused disease.

L. monocytogenes is ubiquitous in the environment and can be found in soil, water, faeces, etc. It has the ability to form biofilms which can contribute to its ability to colonise food processing facilities. It is also resistant to many of the stresses imposed in food processing such as salt (up to 10% salt), temperature (refrigeration temperatures) and detergents (many detergents). Therefore, it can survive in food processing environments and become persistent. Such persistence of *L. monocytogenes* has been shown, often for many years, at larger scale and smaller artisan facilities of different production sectors (Fox et al., 2011; Gomez et al., 2015; Lawrence and Gilmour, 1995; Lomonaco et al., 2009; Ojeniyi et al., 2000; Tocmo et al., 2014).

REGULATIONS RELATING TO *L. MONOCYTOGENES*

In 2005, the Food and Agriculture Organisation and the World Health Organisation (FAO/WHO) held a joint regional conference on food safety for Africa. As a part of this, they identified the agency responsible for food safety in each country and highlighted issues like lack of international standards in food safety legislation, and the need for regional cooperation and collaboration as being important. It was also noted that there is a large degree of underreporting of foodborne illnesses (FAO/WHO, 2005).

In Africa, in general, there is little awareness or regulation relating to *L. monocytogenes*. For example, a recent amendment to the South African Foodstuffs, Cosmetics and Disinfectants Act (1972), referring to microbiological standards has nothing on *Listeria* spp. The Dairy Standard Agency (DSA) has guidelines in its Codes of Practice relating to *L. monocytogenes* in raw milk for final consumption, pasteurised milk, UHT milk, cream and salted butter (DSA, 2012). In these products, the guidelines recommend the absence (in 25 g) of *L. monocytogenes* in raw milk for consumption and in other

products. In general, companies that export, use the relevant regulation in the country they export to. One
South African voluntary standard (South African National Standard [SANS] 885:2011) that specifically refers to the prevalence of *L. monocytogenes* in processed meat products, allows a maximum of 100 cfu/g at the end of shelf-life.

In Europe, Regulation (EC) No 2073/2005 (EC 2005) sets the microbiological criteria for *L. monocytogenes* in foods that must be complied with. This regulation primarily covers RTE food products, and requires that *L. monocytogenes* must be absent from foods (10 x 25 g) intended for infants and for special medical purposes, and allows different criteria depending on the ability of the food product to support growth of *L. monocytogenes.* For RTE foods unable to support the growth of *L. monocytogenes*, the levels should be <100 cfu/g throughout the shelf-life of the product (5 x 25 g). On the other hand, for RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* must not be present in 5 x 25 g samples at the time of leaving the production plant; however, if the producer can show, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout its shelf-life, the level should be <100 cfu/g throughout the shelf life of the product (5 x 25 g).

In Canada (http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php) and Australia/New-Zealand (http://www.foodstandards.gov.au/code/microbiollimits/Pages/Criteria-for-Listeria-monocytogenes-in-ready-to-eat-foods.aspx), the regulations are in line with European regulations, allowing a differentiation between foods that can and cannot support growth.

However, in the USA there is 'zero tolerance' of *L. monocytogenes* (absence in 5 x 25 g of food is required at all times, and in the processing environment), where any occurrence is considered an offence (http://www.fsis.usda.gov/wps/portal/fsis/topics/regulatory-compliance/listeria).

Further discussion on regulations in different jurisdictions is reviewed in a special issue of Food Control published in 2011 (Anonymous, 2011).

OCCURRENCE AND PERSISTENCE OF *L. MONOCYTOGENES*

Occurrence of *L. monocytogenes* in foods and food processing environments

Because *L. monocytogenes* is ubiquitous in the environment and frequently present in the processing environment, it can contaminate food. A number of studies have shown the occurrence of *L. monocytogenes* in foods from Africa and other countries (Table 2). The occurrence of *L. monocytogenes* in some of these due to surveys was relatively high. These high values could be several factors for example, water quality or inadequate hygiene management in companies. Occurrence in/on food is a particular problem if the food can support the growth of the organism.

L. monocytogenes is frequently present in raw foods of both plant and animal origin (including fish), and it can be found in cooked foods due to post-processing contamination. Thus, it has been isolated from foods such as raw and unpasteurized milk, cheese, ice cream, raw vegetables, fermented meats and cooked sausages, raw and cooked poultry, raw meats, and raw and smoked seafood. In addition, its ubiquitous presence also leads to the potential for contamination of the food processing environment, where occurrence and persistence of *L. monocytogenes* is frequent (Nakari et al., 2014; Vongkamjan et al., 2013; Jami et al., 2014).

A number of surveys of *L. monocytogenes* in foods (especially RTE foods) and processing environments within food processing facilities have been performed in recent years. Table 2 shows the frequency of its presence in surveys conducted in Africa. Such surveys give valuable information for particular cases, but tend to be focused on a single analysis time at a few facilities. Surveys conducted over time at several processing facilities provide greater information on the ecology and persistence of *L. monocytogenes.* For instance, in the particular case of Ireland, a study of the occurrence and persistence of *L. monocytogenes* in foods and food processing environments of 48 food businesses involving regular sampling and characterization of isolates by serotyping and pulsed field gel electrophoresis (PFGE) has been recently published (Leong et al., 2014). A European-wide survey on occurrence in different dairy and meat processing facilities over a 12-month period has also been reported (Muhterem-Uyar et al., 2015). Additionally, varying occurrence of *L. monocytogenes* has been reported in smoked fish products and processing facilities (Tocmo et al., 2014; Jami et al., 2014), dairy processing facilities (Pritchard et al. 1995) and ready-to-eat food producing facilities (Kovacevic et al., 2012).

Occurrence of *L. monocytogenes* at retail level

Contamination of RTE foods by *L. monocytogenes* can occur at various stages of the processing and distribution chain, including at retail level, although studies of occurrence at retail level do not necessarily imply that contamination occurred in the retail environment. Cross-contamination with *L. monocytogenes* at retail has been identified as the main source of *L. monocytogenes* in RTE deli products (Sauders et al., 2009; Tompkin, 2002; Vorst et al., 2006). Data from some surveys have indicated that RTE deli products handled at retail level have a significantly higher *L. monocytogenes* prevalence than products pre-packed by the manufacturer and not handled at retail (Gombas et al., 2003). For instance,

Table 2. Occurrence of *Listeria* spp. and *L. monocytogenes* in foods in Africa.

Authors	Country	Foodstuffs/samples	Isolation procedure	Overall prevalence	Prevalence of *L. monocytogenes* by food category
Ajayeoba et al., 2015	Nigeria	RTE vegetables obtained from traditional markets (555 samples)	ISO 11290-1	43.96% *Listeria* spp.	Cabbage (28.28%), Carrot (9.02%), Cucumber (23.36%), Lettuce (19.67%), Tomatoes (19.67%)
Bouayad and Hamdi, 2012	Algeria	RTE dairy and meat foods (227 samples)	AFNOR V08-055	9.3% *Listeria* spp., 2.6% *L. monocytogenes*	Heat-treated dairy products (3%), Meat products (2.6%)
Christison et al., 2008	South Africa	RTE filled baguettes (35), salads (35) from 4 delicatessens	ISO 11290-1	4% *L. monocytogenes*	Filled baguettes (6%), Assorted salads (3%)
Derra et al., 2013	Ethiopia	Retail Meat and Dairy products (240 samples)	International Standard Dairy Federation method	27.5% *Listeria* spp., 4.1% *L. monocytogenes*	Raw meat (6.8%), Raw milk (3.4%), Cottage cheese (1.7%), Cream-cake (5.1%)
El Marnissi et al., 2013	Morocco	Raw milk, Lben (fermented skimmed milk) and Jben (fresh cheese) (288 samples)	ISO 11290-1	5.90% *L. monocytogenes*	Raw milk (8.33%), Lben (5.20%), Jben (4.16%)
Ennaji et al., 2008	Morocco	426 samples: (a) Raw meat (n = 112), meat products (n = 240), poultry (n = 74)	ISO11290, modified	2.4% *L. monocytogenes*	426 samples: Raw meat (3.3%), meat products (0.9%), poultry (1.3%)
El-Shenawy et al., 2011	Egypt	Street vended RTE food (576 samples)	Enrichment in Listeria selective broth (Oxoid) and plated on OXFORD agar	24% *Listeria* spp., 14% *L. monocytogenes*	Meat products (16%), Poultry products (9%), Seafood products (8%), Dairy products (14%), Plant products (24%)
Garedew et al., 2015	Ethiopia	RTE foods of animal origin (384 samples)	ISO 11290-1	25% *Listeria* spp., 6.25% *L. monocytogenes*	Raw meat (6.66%), Minced beef (12%), Fish (6%), Pizza (8.3%), Pasteurized milk (0%), Raw milk (4%), Cottage cheese (0%), Ice cream (15%), Cream cakes (10.7%)
Gebretsadik et al., 2011	Ethiopia	Foods of animal origin (391 samples)	USFDA method	26.1% *Listeria* spp., 5.4% *L. monocytogenes*	Raw milk (13%), Liquid whole egg (4.3%), Raw beef (2.6%), Cottage cheese (1%)
Hakim et al., 2015	Egypt	Retail pork and pork by-products (80 samples)	Enrichment in buffered peptone water, plating on PALCAM	11.25% *L. monocytogenes*	Retail local and imported pork by-products (11.25%)
Ieren et al., 2013	Nigeria	Salad and RTE vegetables (355 samples)	ISO 11290-1	3.9% *L. monocytogenes*	Cabbage (8.5%), Lettuce (6.2%), Coleslaw (4.4%), Convention vegetable salads (1.7%)
Mengesha et al., 2009	Ethiopia	RTE foods and raw meat products (711 samples)	ISO 11290-1	26.6% *Listeria* spp.,4.8% *L. monocytogenes*	Ice cream (11.7%), Cakes (6.5%), Soft cheese (3.9%), Meat products (3.7% to 5.1%)
Morobe et al., 2009	Botswana	Food samples from supermarkets and street vendors (1324 samples)	Enrichment in enrichment broth (Mast diagnostics), plated on Modified Listeria Selective Agar	4.3% *L. monocytogenes*	Cheese (2.75%), Raw milk (1.08%), Meat (biltong) (0%), Frozen cabbage (10.11%), Salad (coleslaw) (7.41%)

Table 2. Contd.

Mugampoza et al., 2011	Uganda	Bulked raw milk, locally processed yoghurt, fermented dairy product (100 samples)	FDA-BAM	6.1% *L. monocytogenes*	Raw milk (13%), Yoghurt (3%), Fermented Diary product (Bongo) (0%)
Salihu et al., 2008	Nigeria	Smoked fish from various retail outlets and market places (115 samples)	ISO 11290-1	25% *L. monocytogenes*	Smoked fish (25%)
van Nierop et al., 2005	South Africa	Fresh and frozen chicken carcasses (99 samples)	Dairy enrichment broth, plating on Oxford agar	19.2% *L. monocytogenes*	Fresh chicken (17%), Frozen chicken (24%)

Gombas et al. (2003) analysed 31,705 samples from retail markets in the USA and found an overall *L. monocytogenes* prevalence of 1.82%, with the prevalence ranging from 0.17 to 4.7% among the product categories tested. Interestingly, these authors observed significantly ($p < 0.001$) higher prevalence for in-store packaged samples than for manufacturer-packaged samples of luncheon meats, deli salads and seafood salads.

It is important to note that recently conducted risk assessments for *L. monocytogenes* in deli meats indicated that the majority of listeriosis cases and deaths associated with deli meats are probably due to contamination of products at retail (Endrikat et al., 2010; Pradhan et al., 2010). Endrikat et al. (2010) estimated that 83% of human listeriosis cases and deaths attributable to deli meats are due to retail-sliced products, and Pradhan et al. (2010) performed a risk assessment using product-specific growth kinetic parameters that indicated that 63 to 84% of human listeriosis deaths linked to deli ham and turkey can be attributed to contamination at retail. Occurrence and cross-contamination at retail level do not attract much research, but are obviously an important source of listeriosis.

Persistence of *L. monocytogenes* in processing environments

The persistence of *L. monocytogenes* in the food-processing environment is well-documented but poorly understood (Carpentier and Cerf 2011; Lomonaco et al., 2009). This is partly due to the loosely defined term “persistence”. Generally, strains of *L. monocytogenes* that have been repeatedly isolated from the same environment over a long period of time for example, over six months, are regarded as being persistent. Persistence of *L. monocytogenes* isolates has been shown, often for many years, at larger scale cheese production facilities (Lomonaco et al., 2009), smaller artisan facilities (Fox et al., 2011), in the salmon industry (Tocmo et al., 2014), in meat processing plants (Gomez et al., 2015) and in poultry production plants (Lawrence and Gilmour, 1995; Ojeniyi et al., 2000). Nevertheless, although it is probable that these strains are surviving and persisting in the food-processing environment, it is also possible that consistent contamination from outside sources, for example, from raw materials, act as a continuous source of particular *L. monocytogenes* strains (Carpentier and Cerf, 2011).

The survival of *L. monocytogenes* in food processing conditions which would be inhospitable to most bacteria can be due to several factors including: (1) ability to grow at a wide range of temperatures, especially refrigeration temperatures (Schmid et al., 2009), (2) resistance to acid stress, (3) resistance to desiccation (Takahashi et al., 2011), (4) resistance to sanitation agents and (5) biofilm formation (Gandhi and Chikindas, 2007; Galvão et al., 2012). This ability to survive where other bacteria cannot, allows *L. monocytogenes* to grow with little competition from other bacteria.

Persistent strains do not appear to have any particular resistance genes to help them survive and persist in the environment, but *L. monocytogenes* strains in general are hardy and resistance to various stresses is commonly seen (Carpentier and Cerf, 2011). These characteristics allow *L. monocytogenes* to survive and possibly even thrive in environments which would be considered unfavourable for general bacterial growth.

A major step to discourage bacterial growth in food processing is storage at refrigeration temperatures of 4°C. Although the majority of food pathogens cannot grow at this temperature, *L. monocytogenes* can. Therefore, refrigerated storage essentially selects for *L. monocytogenes* growth. Cold shock proteins have been shown to be essential for *L. monocytogenes’* ability to survive at low temperature as well as its ability to survive osmotic stress (Schmid et al., 2009). An alternative sigma factor σ^B, encoded by *sigB*, plays a vital role in *L. monocytogenes* stress response. The *sigB* gene has been shown to be vital in the survival of *L. monocytogenes* in prolonged cold storage (Moorhead and Dykes, 2004).

Harbourage sites are also a very important factor in the persistence of *L. monocytogenes.* When used correctly, cleaning and sanitising procedures should be adequate to remove *L. monocytogenes* from the environment (Cruz

and Fletcher, 2012). However, a harbourage site could be an area where sanitation agents do not properly reach so *L. monocytogenes* is not properly removed. When used correctly and in a high enough dosage, *L. monocytogenes* does not seem to have increased resistance to disinfectants as compared to other bacteria (Kastbjerg and Gram, 2012; Lourenço et al., 2009). However, a harbourage site may be an area where the disinfection product reaches but at a lower concentration and it may not be properly dried so that a sub lethal amount of the product remains in the site. This may allow *L. monocytogenes* strains sufficient time to develop a resistance to the product so that a community of *L. monocytogenes* which is resistant to the cleaning product develops. This strain could then be spread out from the harbourage site to contaminate other areas of the facility (Carpentier and Cerf, 2011).

METHODS FOR ANALYSIS OF *L. MONOCYTOGENES*

Methods of detection

L. monocytogenes contamination usually occurs in very low numbers both in foods and in the processing environment so it is vital that any analysis performed includes one or more enrichment steps which inhibit other microflora, and allow both the increase of *L. monocytogenes* in sufficient numbers to allow detection and the recovery of injured/stressed cells. Three methods of analysis are most commonly used: the International Standard (ISO-11290) method which uses a two-step enrichment in Fraser broth, the United States Department of Agriculture (USDA) method which uses a two-step enrichment in University of Vermont media (UVM) and the One-broth Listeria method which has been approved for use by the Association Française de Normalisation (AFNOR) and takes considerably less incubation time and yields results in 2 days as opposed to the 4-5 days needed for the other two methods (Gómez et al., 2013; Zhang et al., 2007). All these methods involve plating on Listeria selective agar (traditional or chromogenic agars) and require confirmation of isolates as *L. monocytogenes* by biochemical or molecular tests.

The use of real-time PCR (RTi-PCR), in combination with traditional culture, to detect the presence or absence of Listeria has also been explored in recent years (Dalmasso et al., 2014; Rossmanith et al., 2010). By amplifying Listeria specific genes through PCR and quantifying them by the detection of a fluorescent probe attached to the DNA fragments, even low numbers of the bacteria can be detected within a few hours (after enrichment) as opposed to the several days it takes to complete traditional plating techniques. For best use, RTi-PCR should be combined with the traditional methods so that isolates can be obtained from the traditional method for strain typing. PCR is not suitable for direct detection of *L. monocytogenes* in food as it lacks the required sensitivity, may be subject to inhibition by food ingredients and can detect the presence of DNA from live as well as dead cells

There is a wide range of different test methods for *Listeria* spp. and *L. monocytogenes* that have been reviewed by Välimaa et al. (2015). These include antibody-based tests, enzyme linked immunosorbent assay (ELISA), immune-capture methods, molecular methods targeting different genes and biosensor methods. Commercial kits are available for many of these methods, but it is not within the scope of this review to give detail of all these methods.

Characterisation of isolates

In order to identify the source or route of contamination, it is necessary to identify the strain type of *L. monocytogenes* contaminating the food or the processing environment rather than just give a positive/negative result. Differentiation of *L. monocytogenes* strains by serotyping is one of the oldest methods of typing and is based on the somatic (O) and flagellar (H) antigen differences between strains. As more exacting typing techniques have since been developed, serotyping of strains now offers little in terms of strain identification but can be helpful in the characterisation of strains (Morobe et al., 2012). Thirteen serotypes are currently recognized which can be broadly split in 4 different serogroups. Doumith et al. (2004) have developed a widely used multiplex PCR which can be used to divide *L. monocytogenes* strains into their serogroup (Doumith et al., 2004). However, to further differentiate strains into their serotype, testing with antisera needs to be performed, which can be prohibitively expensive. Some reactions in antisera testing can be variable, for instance, currently serotypes 4b and 4e cannot be separated by this method. The vast majority of listeriosis outbreaks, approximately 90%, are caused by 1/2b and 4b serotypes, both of which are commonly found in food and food processing facilities. In general, serotype 1/2a has been isolated most frequently from food and the food processing environment (Leong et al., 2014; Shen et al., 2013). Although, it is thought that some serotypes may be generally more virulent than others, currently all *L. monocytogenes* strains must be treated as virulent. Therefore, the identification of certain serotypes in a food or a processing facility does not mean that they will or will not cause disease.

The gold standard for *L. monocytogenes* sub-typing remains pulsed field gel electrophoresis (PFGE), although other methods do offer advantages. PFGE is quite expensive, takes several days and requires trained staff to perform. However, it offers better discriminatory power than most other methods and can be compared between labs if performed according to international

standard practices (PulseNetUSA, 2009). Briefly, PFGE involves the lysis of cells to release the genomic DNA, the immobilisation of the DNA by trapping it in an agarose plug, the restriction digest of the DNA by specific enzymes and the migration of the DNA by gel electrophoresis over a long period of time, generally 21 h. The restriction by a specific restriction digest enzyme gives a distinct pattern of bands, a PFGE pulsotype, which can be used to identify a strain. Generally, two separate restriction digests are performed in two separate PFGE runs which gives a much better differentiation than the use of a single enzyme (Borucki et al., 2004). The resulting PFGE pulsotypes can then be analysed by specialised software in order to accurately compare PFGE pulsotypes and the percentage similarity between strain patterns observed can be calculated. In this way, the same strain found in more than one area of a processing facility or over a period of time can be identified and the likely route/source of contamination may be identified (Strydom et al., 2013).

Sub-typing of isolates, using methods such as pulsed field gel electrophoresis (PFGE), allows analysis of the molecular diversity of *L. monocytogenes* strains present in processing facilities. Strains recurring in the processing environment over time (persistent strains) can be identified (Stessl et al., 2014). Persistent strains in the environment represent an increased risk of contamination of food products. Control of these persistent strains, in particular, is an important part of a food processing facility food safety programme. After characterising the molecular diversity of isolates in the environment in question, putative routes of transmission and/or sources of entry into the environment can be identified. Muhterem-Uyar et al. (2015) identified three potential contamination scenarios that can increase the risk of food contamination, hot-spot contamination (where a specific area is contaminated), widespread contamination (where contamination is spread throughout the facility) and sporadic contamination (where non-persistent contamination occurs on an irregular basis). Visualisation of the contamination on a facility map can help identify the putative contamination routes (Dalmasso and Jordan, 2013). Thus, control strategies can be adjusted/targeted to remove the source of contamination and interrupt the route of transfer to the food. Analysis of such results can not only identify persistent strains, but can also identify an area which may be colonised by a particular strain, leading to possible recontamination events. It can also be used to prevent the spread of strains throughout the facility.

Multilocus sequence typing (MLST) is also commonly used in strain typing, by sequencing a specific set of alleles of housekeeping genes and analysing the variations in the sequences, which allows identification of strain differences. Although less discriminatory than PFGE, the evolutionary distance between strains can be measured, by inspecting the number of alterations in the sequences, which cannot be performed by PFGE (Haase et al., 2014).

PCR to detect different genes present in *L. monocytogenes* strains is also commonly used for strain characterization. The presence/absence of different genes can be a good indication of whether or not a strain is virulent or whether it possesses genes which may help it to persist in a food processing facility. Several genes, such as the stress survival islet SSI-1 and the Tn*6188* transposon, which confers resistance to certain quaternary ammonium compounds, have been identified which appear to confer advantages to strains which may help them to survive in the seemingly inhospitable environment of a processing facility (Müller et al., 2013; Ryan et al., 2010). Similarly, several genes which contribute to virulence have been identified, for example listeriolysin S (LLS) and *actA*, and the use of PCR to detect these genes can help to evaluate strains ability to cause disease (Cotter et al., 2008; Jacquet et al., 2002).

Other options for characterization of *L. monocytogenes* isolates include Multiple-Locus Variable Tandem Repeat Analysis (MLVA), ribotyping, phenotypic or biochemical arrays and Fourier Transform infrared spectroscopy (Stessl et al., 2014).

In recent years, the price of whole genome sequencing (WGS) has lowered significantly allowing the use of WGS in more routine applications. As opposed to PFGE or MLST, WGS examines the entire sequence of a genome, rather than just part of it, and so gives a much higher strain differentiation (Gilmour et al., 2010). Individual genes can also be examined through the use of WGS. For example, in the Quargel cheese outbreak in Austria in 2009/2010, WGS was used to identify 2 distinct 1/2a *L. monocytogenes* strains (QOC1 and QOC2) which overlapped to form the outbreak (Rychli et al., 2014). Through WGS, specific genes which contribute to invasion and survival were also identified including the presence of a *vip* homologue in QOC2 which encodes a surface protein, likely responsible for the higher invasion efficiency of QOC2 in comparison with QOC1. As costs continue to fall, WGS is increasingly being used in outbreak investigations as it offers a much more comprehensive overview of a strain and gives a significantly higher confidence in strain identification.

CHALLENGE STUDIES TO DETERMINE THE ABILITY OF FOOD TO SUPPORT GROWTH OF *L. MONOCYTOGENES*

Certain foods are categorized in a higher risk category for contamination with *L. monocytogenes*. These are ready-to-eat (RTE) foods (including soft cheese, RTE meats and smoked fish), since the heat step of cooking, which would kill any *L. monocytogenes* present, is missing in these foods. Thus, if the food product is able to support the growth of *L. monocytogenes*, bacterial numbers can

reach high levels, even at refrigeration temperatures, posing a health risk for consumers.

Determining the ability of RTE foods to support the growth of *L. monocytogenes* is important, especially in those jurisdictions where there is no “zero tolerance” policy for *L. monocytogenes* (e.g. Europe, Canada and Australia). The ability of *L. monocytogenes* to grow in food products may be estimated based on specifications of the physico-chemical characteristics of the product, consultation of the available scientific literature, or predictive mathematical modelling. There are many tools that support predictive modelling of *L. monocytogenes* in food. These include for example, general pathogen models such as Combase (www.combase.eu) and Pathogen Modelling Programme (PMP; http://pmp.errc.ars.usda.gov/PMPOnline.aspx), and more specific *L. monocytogenes* models such as those at http://safesmokedfish.food.gov.uk/ or http://fssp.food.dtu.dk/. Such predictive models are useful, but for many reasons, including the possibility of overestimation/underestimation of growth in food products, in most cases growth assessment will involve laboratory-based studies, so-called challenge tests. From a public health perspective, overestimation of growth is a ‘fail-safe’ scenario, although such overestimation can be inaccurate from a food producer’s perspective. For example, in 40% of cases Combase predicted growth in cheese when no growth was seen in growth experiments (Schvartzman et al. 2011). It was further shown that the growth characteristics of *L. monocytogenes* were different in liquid and solid matrices (Schvartzman et al., 2010).

A challenge test can be defined as a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable abuse conditions of transportation, storage at retail and at consumer level. Performing challenge tests to assess growth of *L. monocytogenes* on foods is not simple, since different RTE foods may require different laboratory approaches. However, in order to harmonize the laboratory methodology, some agencies have published guidelines in the last decade for the execution of challenge tests. The Food Standards Agency of New Zealand has recently published guidelines for undertaking challenge studies (FSANZ, 2014), although this document is not specifically related to *L. monocytogenes*. On the other hand, Canada also has guidelines which specifically relate to *L. monocytogenes* (Health Canada, 2012). In Europe, in order to facilitate the task of performing challenge studies, the European Union Community Reference Laboratory for *L. monocytogenes* (EURL *Lm*) prepared a Technical Guidance document in 2008 (EC, 2008). This guidance document, which was aimed at describing the microbiological procedures for determining growth of *L. monocytogenes* using challenge tests in the frame of the application of Regulation (EC) No. 2073/2005, has been recently updated (EC, 2014). The European Guidance document of 2014, recently reviewed by Alvarez-Ordóñez et al. (2015), helps the Food Business Operator to decide whether a challenge test would be required for their food product, and describes the laboratory methodology that must be followed when carrying out a challenge test. This guidance document differentiates two types of challenge tests: the ones that determine growth potential of an inoculated strain or strains and those that calculate the growth rate of the strain(s). Growth potential is defined as the difference between the $\log_{10}$ cfu/g at the end of the shelf-life and the $\log_{10}$ cfu/g at the beginning of the test. When this difference is greater than 0.5 $\log_{10}$ cfu/g, the food is classified into RTE foods that are able to support the growth of *L. monocytogenes.* Alternatively, when the difference is less than 0.5 $\log_{10}$ cfu/g, the food is classified into RTE foods that are unable to support the growth of *L. monocytogenes*. The growth rate is on the other hand calculated from the growth curve as the slope of the straight line resulting from plotting the $\log_{10}$ of cell numbers against time in the exponential phase of growth. The growth rate is an important parameter of the growth curve which depends on the inoculated strain(s), the intrinsic properties of the food (e.g. pH, NaCl content, aw, associated microflora, antimicrobial constituents), and extrinsic properties (e.g. temperature, gas atmosphere, moisture). Once the growth rate is known for a given food at a given temperature, it is possible to estimate the concentration of *L. monocytogenes* at a given day of the shelf-life if the initial concentration is known. It is also possible to extrapolate the growth rate at a given temperature to predict growth rates at other temperatures in the same food.

Control of *L. monocytogenes*

As *L. monocytogenes* is an ubiquitous organism, its complete elimination is an unrealistic aim. Control is a more practical approach. Such control can be achieved by attention to detail in hygiene strategies, monitoring occurrence of the organism or using novel control methods such as bacteriocins and bacteriophage.

Novel methods of control

In recent years, in addition to novel technologies such as high pressure processing and pulsed electric field, novel methods for control of pathogens (and spoilage organisms) has focused on the use of natural anti microbial agents such as bacteriocins and bacteriophage.

Bacteriocins

Bacteriocins are ribosomally-synthesised peptides that are pore-forming agents, which act by disrupting the integrity of the target cell membrane. They have the

potential to inhibit other bacteria, including pathogens, in many cases resulting in cell death. Therefore, they have potential as a mechanism to control *L. monocytogenes*. The spectrum of activity can be broad, where a wide variety of unrelated species are inactivated, or narrow, where only closely related species are inactivated. To date, insufficient data has been generated to obtain a complete picture of the potential use for many bacteriocins. The current regulatory situation dictates against the use of bacteriocins as biocontrol agents as in many cases, there is currently insufficient supporting data to assure the regulatory authorities of their efficacy and safety (Cotter et al., 2013).

Bacteriophage

Bacteriophages are viruses that infect and can kill bacteria and are logical candidates for biocontrol of *L. monocytogenes* in food. They exhibit a high degree of specificity towards their target host bacterium, and as a result, are safe for use in food processing, considering they will have no detrimental effect on the microflora of the eventual consumer, nor will they have an effect on any other desired bacteria in the food. They also have other desirable attributes, including a relative stability during storage, and the ability to self-perpetuate. Of particular importance in terms of suitability for biocontrol of *L. monocytogenes* is finding a virulent bacteriophage phage that is strictly lytic, rather than a lysogenic phage which can be genetically unstable. Lytic phages are genetically stable, will always kill infected cells, and cannot therefore integrate its genome into that of the bacterial chromosome. It is also of critical importance that the full genome sequence of such phage is known, and that any phage applied to food does not encode any virulence factors or toxins which may be harmful (Hagens and Loessner, 2010).

The consensus among microbiologists is that bacteriophages do not have any known adverse effects on humans, animals or the environment. For this reason, many scientists and food safety experts predict that bacteriophages could become a useful tool in the reduction of pathogens in the food chain. However, there are concerns that limited safety data testing has been undertaken, although bacteriophages have been widely used for treatment of human diseases in the former Soviet Union (Chanishvili, 2012).

The renewed interest in the use of bacteriophage as biocontrol agents has resulted in the development of several commercial products designed for this purpose, such as LMP-102 phage preparation (now more commonly known as ListShield™) and Listex™. Although products have been approved for use in some countries, their use is not permitted in others. Biocontrol of *L. monocytogenes* with bacteriophage was reviewed by Strydom and Witthuhn (2015).

National monitoring programmes

Monitoring the food processing environment for the presence of *L. monocytogenes* can be an effective mechanism in its control (Dalmasso and Jordan, 2013). Indeed, EU regulations require that food processing environments are sampled, although they do not state the number of samples to be taken, or the frequency of sampling (EC, 2005).

In South Africa, over the last decade, most of the major retailers have developed their own food safety standards and audit protocols in order to protect their brands, and ultimately the consumer. These standards are all based on national legal requirements, for example, regulation R692 governing microbiological standards for foodstuffs and related matters (Foodstuffs, Cosmetics and Disinfectants Act, 1972) and prerequisite programmes as defined by the voluntary national standards of the South African Bureau of Standards. These regulations apply to a wide range of foodstuffs and beverages and while the absence of specified genera and species of various pathogenic bacteria are required in the products mentioned in these regulations, *Listeria* was not mentioned. Similarly, in regulation 1555 relating to milk and dairy products, all pathogens are required to be absent from raw milk intended for further processing or consumption, with no specific mention of *Listeria.* In an attempt to rationalise the number of audits and create a national approach to a food safety management system, the Consumer Goods Council of South Africa (CGCSA) formed the Food Safety Initiative (FSI) to promote a single audit standard. As all major retailers are members of a similar international organisation known as the Global Food Safety Initiative (GFSI), the decision was taken to adopt the GFSI Global Markets Capacity Programme as the single audit standard. The GFSI Global Markets Programme was launched in 2008 by the Global Food Safety Initiative to help small or less developed companies achieve certification to GFSI recognised food safety schemes and market access. It also helps to build food safety capacity through a structured, step-by-step approach.

From a dairy perspective, the DSA is a non-profit making company that aims to promote the compliance of milk and other dairy products, on a national basis, with product composition, food safety and metrology standards. This is done by regular and systematic monitoring of dairy products on farms and on retail shelves. In the DSA Codes of Practice (Milk South Africa, 2015), guidelines recommend the absence (in 25 g of product) of *L. monocytogenes* in raw milk for human consumption, pasteurised milk, UHT milk, cream and salted butter.

In Austria, a National Monitoring Programme has been established on a voluntary basis in the cheese industry. This is aimed at early detection of *L. monocytogenes* followed by targeted intervention strategies. There are

four levels of investigation; Level 1 deals with the routine monitoring of samples, Level 2 is an intervention phase if positive results are detected, Level 3 is an intensive sanitation phase and Level 4 is a verification phase to confirm successful control.

In the Republic of Ireland, a research project on Listeria monitoring in food processing environments commenced in March 2013. Sixty seven food businesses, categorised into several industry sectors, such as dairy, meat, fish and vegetables, were involved in the project. Every two months, each business submits six environmental swab samples and two food samples for analysis by the ISO 11290 method. Businesses are informed on presumptive results immediately so that corrective actions can be taken, if necessary. Confirmatory PCR, serotyping and PFGE are performed on all isolates obtained. PFGE allows the identification of persistent strains and businesses are offered advice especially if particular contamination issues (such as persistence) are identified. Through this programme, a pattern of contamination in Irish food processing facilities can be seen, and a general *L. monocytogenes* contamination level of 4.6% was found in the first year of the programme with a similar positive percentage found in food and environmental samples (Leong et al., 2014).

A similar programme of monitoring has recently been established in Northern Ireland.

Control of *L. monocytogenes* in the processing environment

It is relatively difficult to maintain a completely L. monocytogenes-free processing environment as many varying factors can have an effect on the occurrence of *L. monocytogenes* in the processing facility. These can include, for example, contaminated incoming raw materials, staff members acting as *L. monocytogenes* carriers, inefficient cleaning strategies and sampling programmes in place, the facility design to prevent contamination, the location of the facility near a farm, etc. Another major factor in the occurrence of *L. monocytogenes* is the awareness of the processing facility management and staff. The operation of a processing facility requires constant vigilance against bacterial contamination through various methods, and lack of awareness in this area can lead to more significant problems in end products which can result in product recalls, damage to company reputation, lawsuits, illnesses or even death. Thus, sampling and analysis are key factors in successful control. If occurrence is detected it can be eliminated through targeted intervention measures that help to prevent product contamination.

Although, final product testing is important in *L. monocytogenes* control programmes, it does not give information on the source and routes of product contamination. On the other hand, environmental testing is a more effective way to monitor hygiene and prevent contamination events (Tompkin, 2002). Tracing the source of *L. monocytogenes* is critical in the control of the organism in a localised environment, although the ubiquitous nature of *L. monocytogenes* makes it difficult to positively identify the source of contamination in some occasions. The potentially long incubation time for *L. monocytogenes* to cause disease can also make it difficult to trace the disease to a specific food and source of contamination (Goulet et al., 2013). It is therefore important to remove as many sources of contamination as possible from the food processing environment to reduce the possibility of food contamination.

Of utmost importance when sampling a processing environment for *L. monocytogenes* is actively looking for it, as opposed to selecting for negative results in order to adhere to regulations. Sampling directly after disinfection or cleaning or sanitation, for example, should be discouraged, unless the sampling is being used to evaluate the efficacy of the cleaning procedures. Proper sampling of a processing environment should include several areas in which contamination is most likely to occur, including both food contact and non-food contact surfaces. One of the most common areas to be contaminated are floor drains as any contamination throughout the facility is likely to be washed through the drain where *L. monocytogenes* can persist in a harbourage site (Carpentier and Cerf, 2011). Sampling should be done with a sponge-type swab, allowing sufficient surface area to be sampled. Adequate sampling will allow problems of contamination to be pre-empted and addressed in a timely manner. *L. monocytogenes* contamination of food products is a much more serious problem which requires significantly more intervention than contamination at the processing stage.

The following guidelines may help in tackling problems with L. monocytogenes.

Understanding the nature of *L. monocytogenes* contamination and attaching importance to it

Most food processing environments are contaminated to some extent. Adequate sampling for *L. monocytogenes* will help identify issues, which should be addressed immediately.

Regulations should be taken seriously and a food processing environment monitoring plan developed as a core activity of good hygiene practices (GHP).

Choosing the right sampling sites and methodology

The processing environment should be sampled with a view to finding the organism. The most informative sampling sites can vary depending on the food commodity

produced. The difference in information that will be gained from sampling of food contact materials versus non-food contact materials should be considered. Sampling is the most critical procedural step and, if done inappropriately is of little benefit. Swabs that have enough contact surface to sample the 900 cm2 mentioned in many guidelines should be used. Sampling sites from manufacturing or handling steps that are applied on most of the products produced should be chosen (e.g. conveyor belts before packaging, slicer blades, etc.).

Choosing the right sampling frequency

Recommendations on sampling frequency can only be expressed in general terms. If a food processing environment (FPE) is being sampled for the first time, a broad sampling approach is used. If the contamination status is already known, a restricted number of sampling sites should be tested frequently rather than a lot of sampling sites only once. Sampling frequency can be reduced if negative results are shown, but should be increased again if positive results are detected or if there are changes to the processing environment or manufacturing process. Sampling frequency should be dynamic.

Establishing critical control areas

Prioritisation of counter-measures, clearly defining critical control areas (CCA) where FPE contamination is not acceptable under any circumstances should be facilitated. It makes a difference whether a *L. monocytogenes* positive drain is located in a general processing area or if it is located where food is handled prior to packing. Critical control areas should be clearly marked (e.g. by marks on floors, in construction maps) and hygiene barriers should prevent CCAs from being visited or trespassed by unqualified personnel. Hygiene barriers, such as footbaths and change of personal protective clothing should reduce the risk of cross-contamination with *L. monocytogenes*. The high hygiene standard that should exist in CCAs can only be monitored by taking an appropriate number of FPE samples.

Trace the route of transmission of isolates most importantly in CCAs

To combat contamination, it is vital to keep all isolates at a safe and appropriate place (e.g. a contract laboratory). Use molecular typing to identify the putative routes of transmission of a pathogen in the facility, if possible. To reduce the costs, start with combating contamination in a CCA where the risk for contamination of the food commodity is the highest.

Be particularly aware at times of construction

During building work, hygiene measures are usually difficult to maintain at a food processing facility. On the one hand, craftsmen of various occupations with no training in hygiene need to have access to the FPE. Recommending the use of hygiene protection (overshoes and overcoats) to craftsmen is frequently in vain because it limits their maneuverability. Building material, often stored outdoors before use, needs to be carried around. Insects and rodents can get access to the FPE. On the other hand, the food business operator (FBO) frequently needs to produce food in processing rooms adjacent to the construction area. Be aware of the increased risk of cross contamination during such construction periods, and construct physical barriers between food production and construction. The FBO should try to prevent access of craftsmen to production areas as much as possible. Careful and intensified sanitation programmes in the processing areas during the construction phase, and sanitisation of the entire FPE after completion of the construction phase should be observed. The success of this process should be verified by subsequent sampling of the FPE.

Critical review of the floor sanitation procedures applied in cases of widespread contamination

If FPE monitoring demonstrates a widespread contamination of a genetically indistinguishable *L. monocytogenes* strain, sanitation procedure (specify the type of sanitizer to be used and use it appropriately. Make sure all areas are covered. Allow all the surfaces to dry off before food), and the workflow system should be re-considered. Drain water sampling should be used to control the efficiency of sanitation.

Structuring your data and using a processing facility map (roughly drawn) to document your progress and efforts

Safe food production is possible even if there is contamination of a FPE. However, the following criteria must be met:

1. The extent of contamination must be known (implies intensified sampling)
2. Contamination must be never detected in the food commodity produced
3. FPE contamination must be infrequent (reported only irregularly)
4. Contamination must be detectable only in compartments where the risk for cross-contamination is low
5. The food produced must not support growth of *L. monocytogenes* on its surface.

Documentation is critical in any FBO communication process, either within an operation or with regulators or specialists from the outside. Documentation of ingredients and raw materials used as well as any contamination patterns is essential. A map of the facility (roughly drawn) can help with this.

To demonstrate that the FBO has met these requirements, is necessary to organize the data into a structured decision making process. The advice of experts that help to facilitate the decision making process should be sought.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Ajayeoba TA, Atanda OO, Obadina AO, Bankole MO, Adelowo OO (2015). The incidence and distribution of *Listeria monocytogenes* in ready-to-eat vegetables in South-Western Nigeria. Food Sci. Nut. doi:10.1002/fsn3.263.

Álvarez-Ordóñez A, Leong D, Hickey B, Beaufort A, Jordan K (2015). The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. Food Res. Int. 75:233-243.

Anonymous (2011). Listeria Regulation. Food Control 22(9):1477-1549.

Anonymous (2015). Annual Report on Zoonoses in Denmark 2014, National Food Institute, Technical University of Denmark. Available at: http://www.food.dtu.dk/-/media/Institutter/Foedevareinstituttet/Publikationer/Pub-2015/Annual%20Report%202014%20final.ashx?la=da. Accessed 21/10/2015.

Borucki MK, Kim SH, Call DR, Smole SC, Pagotto F (2004). Selective discrimination of *Listeria monocytogenes* epidemic strains by a mixed-genome DNA microarray compared to discrimination by pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing. J. Clin. Microbiol. 42(11):5270-5276.

Bouayad L, Hamdi T-M (2012). Prevalence of *Listeria* spp. in ready to eat foods (RTE) from Algiers (Algeria). Food Control 23(2):397-399.

Carpentier B, Cerf O (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. International J. Food Microbiol. 145(1):1-8.

CDC (2011). Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado, Final report. Available at: http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/120811/index.html Accessed 27/10/2015.

CDC (2012). Multistate Outbreak of Listeriosis Linked to Imported Frescolina Marte Brand Ricotta Salata Cheese Available at: http://www.cdc.gov/listeria/outbreaks/cheese-09-12/index.html Accessed 16/1/2015.

CDC (2013). Multistate Outbreak of Listeriosis Linked to Crave Brothers Farmstead Cheeses Available at: http://www.cdc.gov/listeria/outbreaks/cheese-07-13/index.html Accessed 16/1/2015.

CDC (2014a). Multistate Outbreak of Listeriosis Linked to Commercially Produced, Prepackaged Caramel Apples In. http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html Accessed 16/1/2015.

CDC (2014b). Multistate Outbreak of Listeriosis Linked to Roos Foods Dairy Products Available at: http://www.cdc.gov/listeria/outbreaks/cheese-02-14/index.html Accessed 16/1/2015.

CDC (2015a). Multistate Outbreak of Listeriosis Linked to Commercially Produced, Prepackaged Caramel Apples Made from Bidart Bros. Apples (Final Update). Available at: http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/ Accessed 21/10/ 2015.

CDC (2015b). Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update) http://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/. Accessed 21/10/2015.

Chanishvili N (2012). A Literature Review of the Practical Application of Bacteriophage Research. Nova Biomedical Books.

Christison C, Lindsay D, Von Holy A (2008). Microbiological survey of ready-to-eat foods and associated preparation surfaces in retail delicatessens, Johannesburg, South Africa. Food Control 19:727-733.

Cotter PD, Draper LA, Lawton EM, Daly KM, Groeger DS, Casey PG, Ross RP, Hill C (2008). Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes*. PLoS Pathog 4(9):e1000144.

Cotter PD, Ross RP, Hill C (2013). Bacteriocins — a viable alternative to antibiotics? Nature Rev. Microbiol. 11:95-105.

Cruz CD, Fletcher GC (2012). Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. Food Control 26:194-199.

Dairy Standards Agency (2012). Available at: http://www.dairystandard.co.za/. Accessed 28/10/2015.

Dalmasso M, et al. (2014). Comparison of polymerase chain reaction methods and plating for analysis of enriched cultures of *Listeria monocytogenes* when using the ISO11290-1 method. J. Microbiol. Methods 98:8-14.

Dalmasso M, Jordan K (2013). Process environment sampling can help to reduce the occurrence of *Listeria monocytogenes* in food processing facilities. Irish J. Agric. Food Res. 52:93-100.

de Castro V, Escudero JM, Rodriguez JL, Muniozguren N, Uribarri J, Saez D, Vazquez J (2012) Listeriosis outbreak caused by Latin-style fresh cheese, Bizkaia, Spain, August 2012. Euro surveillance: European Communicable Disease Bulletin 17(42).

de Noordhout CM, Develeesschauwer B, Angulo FJ, Verbeke G, Haagsma J, Kirk M, Havelaar A, Speybroeck N (2014). The global burden of listeriosis: a systematic review and meta-analysis. The Lancet Infectious Diseases 14(11):1073-1082.

Derra FA, Kalsmose S, Monga DP, Mache A, Svedsen CA, Félix B, Granier SA, Geyid A, Taye G, Hendriksen RS (2013). Occurrence of *Listeria* spp. in retail meat and dairy products in the area of Addis Ababa, Ethiopia. Foodborne Path. Dis. 10:577-579.

Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. 42:3819-3822.

EC (2005). COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union L338(3).

EC (2008). Technical Guidance Document on shelf-life studies for *Listera moncytogenes* in ready-to eat foods. Available at: http://ec.europa.eu/food/food/biosafety/salmonella/docs/technical_guidance_listeria_en.pdf Accessed 19/1/2015.

EC (2014). EURL Lm TECHNICAL GUIDANCE DOCUMENT for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods. Available at http://ec.europa.eu/food/food/biosafety/salmonella/docs/technical_guidance_listeria_en.pdf Accessed 19/1/2015.

El Marnissi B, Bennani L, Cohen N, Lalami AEO, Belkhou R (2013).

Presence of *Listeria monocytogenes* in raw milk and traditional dairy products marketed in the north-central region of Morocco. African J. Food Sci. 7:87-91.

El-Shenawy M, El-Shenawy M, Manes J, Soriano JM (2011). *Listeria* spp. in street-vended ready-to-eat foods. Interdisciplinary perspectives on infectious diseases 2011. doi:10.1155/2011/968031.

Endrikat S, Gallagher D, Pouillot R, Hicks Quesenberry H, Labarre D, Schroesder CM, Kause J (2010). A comparative risk assessment for *Listeria monocytogenes* in prepackaged versus retail-sliced deli meat. J. Food Prot. 73:612-619.

Ennaji H, Timinouni M, Ennaji M, Hassar M, Cohen N (2008). Characterization and antibiotic susceptibility of *Listeria monocytogenes* isolated from poultry and red meat in Morocco. Infect. Drug Resist. 1:45-50.

FAO/WHO (2005). National Food Safety Systems in Africa – A Situation Analysis, prepared for the FAO/WHO Regional Conference of Food Safety for Africa, October 2005 (CAF 05/2).

Food Safety Australia New Zealand (2014). How to Determine the Shelf Life of Food: A Guidance Document (Food Standards Agency New Zealand). Available at: http://www.foodsafety.govt.nz/elibrary/industry/determine-shelf-life-of-food/how-to-determine-the-shelf-life-of-food-revision.pdf Accessed 6/10/2014.

Foodstuffs, cosmetics and disinfectants (1972). Foodstuffs, cosmetics and disinfectants act 54 of 1972. Available at: http://www.nda.agric.za/vetweb/Legislation/Other%20acts/Act%20-%20Foodstuffs,%20Cosmetics%20and%20Disinfectants%20Act-54%20of%201972.pdf. Accessed 23/10/2015.

Fox E, Hunt K, O'Brien M, Jordan K (2011). *Listeria monocytogenes* in Irish Farmhouse cheese processing environments. Int. J. Food Microbiol. 145, Supplement 1:S39-S45.

Fredericks P, Britz M, Eastman R, Carr JA, Bateman KJ (2015). Listerial brainstem encephalitis--treatable, but easily missed. South African Med. J. 105:17-20.

Fretz R, Pichler J, Sagel U, Much P, Ruppitsch W, Pietzka AT, Stöger A, Huhulescu S, Heuberger S, Appl G, Werber D, Stark K, Prager R, Flieger A, Karpíšková R, Pfaff G, Allenberger F (2010). Update: Multinational listeriosis outbreak due to 'Quargel', a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009-2010. Euro surveillance: European Communicable Disease Bulletin 15(16).

Galvão NN, Chiarini E, Destro MT, de Aguiar Ferreira M, Nero LA (2012). PFGE characterisation and adhesion ability of *Listeria monocytogenes* isolates obtained from bovine carcasses and beef processing facilities. Meat Sci. 92:635-643.

Gandhi M, Chikindas ML (2007). Listeria: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113:1-15.

Garedew L, Taddese A, Biru T, Nigatu S, Kebede E, Ejo M, Fikru A, Birhanu T (2015). Prevalence and antimicrobial susceptibility profile of *Listeria* species from ready-to-eat foods of animal origin in Gondar Town, Ethiopia. BMC Microbiol. 15:100. doi:10.1186/s12866-015-0434-4.

Gebretsadik S, Kassa T, Alemayehu H, Huruy K, Kebede N (2011). Isolation and characterization of *Listeria monocytogenes* and other *Listeria* species in foods of animal origin in Addis Ababa, Ethiopia. J. Infect. Public Health 4:22-29.

Gilmour MW, Graham M, Van Domselaar G, Tyler S, Kent H, Trout-Yakel KM, Larios O, Allen V, Lee B, Nadon C.(2010). High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genom. 11:120 doi:10.1186/1471-2164-11-120.

Gombas DE, Chen Y, Clavero RS, Scott VN (2003). Survey of *Listeria monocytogenes* in ready-to-eat foods. J. Food Prot. 66:559-569.

Gómez D, McGuinness S, Reddington K, O'Grady J, Yangüela J, Barry T (2013). Evaluation of a novel Listeria enrichment broth combined with a real-time PCR diagnostics assay for the specific detection of *Listeria monocytogenes* in RTE pork products. Int. J. Food Sci. Technol. 48:1103-1108.

Gomez D, Pilar Iguacel L, Rota M, Carraminana JJ, Arino A, Yanguela J (2015). Occurrence of *Listeria monocytogenes* in Ready-to-Eat Meat Products and Meat Processing Plants in Spain, Foods 4:271-282.

Goulet V, King LA, Vaillant V, de Valk H (2013). What is the incubation period for listeriosis? BMC Infect. Dis. 13:11. doi:10.1186/1471-2334-13-11.

Guillet C, Join-Lambert O, Le Monnier A, Leclercq A, Mechai F, Mamzer-Bruneel M, Bielecka MK, Scortti M, Disson O, Berche P, Vazquez-Boland J, Lortholary O, Lecuit M (2010). Human Listeriosis Caused by *Listeria ivanovii*. Emerg. Infect. Dis. 16:136-138.

Haase JK, Didelot X, Lecuit M, Korkeala H, Achtman M (2014). The ubiquitous nature of *Listeria monocytogenes* clones: a large-scale Multilocus Sequence Typing study. Environ. Microbiol. 16:405-416.

Hagens S, Loessner MJ (2010). Bacteriophage for biocontrol of foodborne pathogens: Calculations and considerations. Curr. Pharma. Biotechnol. 11:58-68.

Hakim AS, Abuelnaga AS, Ezz-Eldeen AM, Bakry MA, Ismail SA (2015). Prevalence of some food poisoning bacteria in local and imported retail pork by-products in Egyptian markets. African J. Microbiol Res. 9:1492-1498.

Health Canada (2012). *Listeria monocytogenes* Challenge Testing of Refrigerated Ready-to-Eat Foods. Available at: http://www.hc-sc.gc.ca/fnan/alt_formats/pdf/legislation/pol/listeria_monocytogenes-test-eng.pdf Accessed 19/1/2015.

Ieren I, Bello M, Kwaga J (2013). Occurrence and antibiotic resistance profile of *Listeria monocytogenes* in salad vegetables and vegetable salads sold in Zaria, Nigeria. Afr. J. Food Sci. 7:334-338.

Jacquet C, Gouin E, Jeannel D, Cossart P, Rocourt J (2002). Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. Appl. Environ. Microbiol. 68:616-622.

Jami M, Ghanbari M, Zunabovic M, Domig, K J, Kneifel W (2014). *Listeria monocytogenes* in Aquatic Food Products-A Review. Comp. Rev Food Sci. Food Safety 13:798-813.

Kastbjerg VG, Gram L (2012). Industrial disinfectants do not select for resistance in *Listeria monocytogenes* following long term exposure. Int. J. Food Microbiol. 160:11-15.

Kovačević J, McIntyre LF, Henderson SB, Kosatsky T (2012). Occurrence and distribution of listeria species in facilities producing ready-to-eat foods in British Columbia, Canada. J. Food Prot. 75:216-24.

Lawrence LM, Gilmour A (1995). Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. Appl. Environ. Microbiol. 61:2139-2144.

Leong D, Alvarez-Ordonez A, Jordan K (2014). Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. Front. Microbiol. 5 doi:10.3389/fmicb.2014.00436.

Lomonaco S, Decastelli L, Nucera D, Gallina S, Manila Bianchi D, Civera T (2009). *Listeria monocytogenes* in Gorgonzola: Subtypes, diversity and persistence over time. Int. J. Food Microbiol. 128:516-520.

Lourenço A, Neves E, Brito L (2009) Susceptibility of *Listeria monocytogenes* from traditional cheese-dairies to in-use sanitizers. Food Control 20:585-589.

Mengesha D, Molla Zewde B, Toquin M-T, Kleer J, Hildebrandt G, Gebreyes WA (2009). Occurrence and Distribution of *Listeria monocytogenes* and Other Listeria Species in Ready-to-Eat and Raw Meat Products. Berl. Munch. Tierarztl. Wochenschr. 15:20-34.

Milk South Africa (2015). Milk Essay, volume 6 number 2. Available at: http://www.milksa.co.za/sites/default/files/milk_essay/MilkSA_Essay_September.pd. Accessed 23/10/2015.

Moorhead SM, Dykes GA (2004). Influence of the sigB gene on the cold stress survival and subsequent recovery of two *Listeria monocytogenes* serotypes. Int. J. Food Microbiol. 91:63-72.

Morobe I, Gashe B, Obi C, Nyila M, Matsheka M (2012). Molecular Characterization and Serotyping of *Listeria monocytogenes* with a Focus on Food Safety and Disease Prevention. INTECH Open Access Publisher.

Morobe I, Obi C, Nyila M, Gashe B, Matsheka M (2009). Prevalence, antimicrobial resistance profiles of *Listeria monocytognes* from various foods in Gaborone, Botswana. African J. Biotechnol. 8:6383-6387.

Mugampoza D, Muyanja C, Ogwok P, Serunjogi M, Nasinyama G

(2011). Occurrence of *Listeria monocytogenes* in bulked raw milk and traditionally fermented dairy products in Uganda. African J. Food, Ag, Nut. Dev. 11:4610-4622.

Muhterem-Uyar M, Dalmasso M, Bolocan AS, Hernandez M, Kapetanakou AE, Kuthta T, Manios SG, Melero B, Minarovičová J, Nicolau AI, Rovira J, Skandamis PN, Jordan K, Rodríguez-Lázaro D, Stessl B, Wagner M (2015). Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. Food Control 51:94-107.

Müller A, Rychli K, Muhterem-Uyar M, Zaiser A, Stessl B, Guinane CM, Cotter PD, Wagner M, Schmitz-Esser S (2013). Tn6188 - A Novel Transposon in *Listeria monocytogenes* Responsible for Tolerance to Benzalkonium Chloride. PLoS ONE 8(10):e76835.

Nakari UM, Rantala L, Pihlajasaari A, Toikkanen S, Johansson T, Hellsten C, Raulo SM, Kuusi M, Siitonen A, Rimhanen-Finne R (2014). Investigation of increased listeriosis revealed two fishery production plants with persistent *Listeria* contamination in Finland in 2010. Epidemiol. Infect. 142:2261-2269.

Ojeniyi B, Christensen J, Bisgaard M (2000). Comparative investigations of *Listeria monocytogenes* isolated from a turkey processing plant, turkey products, and from human cases of listeriosis in Denmark. Epidemiol. Infect. 125:303-308.

Pradhan AK, Ivanek R, Gröhn YT, Bukowski R, Geornaras I, Sofos JN, Wiedmann M (2010). Quantitative risk assessment of listeriosis-associated deaths due to *Listeria monocytogenes* contamination of deli meats originating from manufacture and retail. J. Food Prot. 73:620-630.

Pritchard TJ, Flanders KJ, Donnelly CW (1995). Comparison of the incidence of *Listeria* on equipment versus environmental sites within dairy processing plants. Int. J. Food Microbiol. 26:375-384.

PulseNetUSA (2009). International Standard PulseNet protocol. Available at: http://www.cdc.gov/pulsenet/pathogens/listeria.html Accessed 23/4/2014.

Rossmanith P, Mester P, Wagner M, Schoder D (2010). Demonstration of the effective performance of a combined enrichment/real-time PCR method targeting the prfA gene of *Listeria monocytogenes* by testing fresh naturally contaminated acid curd cheese. Lett. Appl. Microbiol. 51:480-484.

Ryan S, Begley M, Hill C, Gahan CG (2010). A five-gene stress survival islet (SSI-1) that contributes to the growth of *Listeria monocytogenes* in suboptimal conditions. J. Appl. Microbiol 109:984-95 doi:10.1111/j.1365-2672.2010.04726.x.

Rychli K, Müller A, Zaiser A, Schoder D, Allerberger F, Wagner M, Scmitz-Esser S (2014). Genome sequencing of *Listeria monocytogenes* "Quargel" listeriosis outbreak strains reveals two different strains with distinct *in vitro* virulence potential. PloS one 9(2):e89964 doi:10.1371/journal.pone.0089964.

Salihu M, Junaidu AU, Manga SB, Gulumbe ML, Magaji AA, Ahmed A, Adamu AY, Shittu A, Balarabe I (2008). Occurrence of *Listeria monocytogenes* in smoked fish in Sokoto, Nigeria. Afr. J. Biotechnol. 7:3082-3084.

Sauders BD, Sanchez MD, Rice DH, Corby J, Stich S, Fortes ED, Roof SE, Wiedmann M (2009). Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J. Food Prot. 72:2337-2349.

Scallan E, et al. (2011). Foodborne illness acquired in the United States--major pathogens. Emerg. Infect. Dis. 17:7-15.

Schlech WF (2000). Foodborne listeriosis. Clin. Infect. Dis. 31: 770-775.

Schmid B, Klumpp J, Raimann E, Loessner MJ, Stephan R, Tasara T (2009). Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl. Environ. Microbiol. 75:1621-1627.

Schvartzman MS, Belessi C, Butler F, Skandamis PN, Jordan KN (2011). Effect of pH and water activity on the growth limits of *Listeria monocytogenes* in a cheese matrix at two contamination levels. J. Food Prot. 74:1805-1813.

Schvartzman MS, Belessi X, Butler F, Skandamis P, Jordan K (2010). Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. J. Appl. Microbiol. 109:1790-1799.

Shen J, Rump L, Zhang Y, Chen Y, Wang X, Meng J (2013). Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. Food Microbiol. 35:58-64.

South African National Standards [SANS] (2011) 885: Edition 3 of 2011. South African Bureau of Standards.

Stessl B, Fricker M, Fox E, Karpiskova R, Demerova K, Jordan K, Ehling-Schulz M, Wagner M (2014). Collaborative survey on the colonization of different types of cheese-processing facilities with *Listeria monocytogenes*. Foodborne Path. Dis. 11:8-14.

Strydom A, Bester IM, Cameron M, Franz CM, Witthuhn RC (2013). Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE. Food Control 31:274-279.

Strydom A, Witthuhn CR (2015). *Listeria monocytogenes*: A Target for Bacteriophage Biocontrol. Comp. Rev. Food Sci. Food Safety 14:694-704.

Takahashi H, Kuramoto S, Miya S, Kimura B (2011). Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. Food Control 22:633-637.

Tocmo R, Krizman K, Khoo WJ, Phua, LK, Kim M, Yuk H-G (2014). *Listeria monocytogenes* in Vacuum-Packed Smoked Fish Products: Occurrence, Routes of Contamination, and Potential Intervention Measures. Comp. Rev. Food Sci. Food Safety 13:1735-1739.

Tompkin RB (2002). Control of *Listeria monocytogenes* in the food-processing environment. J. Food Prot. 65:709-725.

Valimaa AL, Tilsala-Timisjarvi A, Virtanen E (2015). Rapid detection and identification methods for *Listeria monocytogenes* in the food chain – A review. Food Control 55:103-114.

van Nierop W, Dusé AG, Marais E, Aithma N, Thothobolo N, Kassel M, Stewart R, Potgieter A, Fernandes B, Galpin JS, Bloomfield SF (2005). Contamination of chicken carcasses in Gauteng, South Africa, by *Salmonella*, *Listeria monocytogenes* and *Campylobacter*. Int. J. Food Microbiol. 99:1-6.

Vongkamjan K, Roof S, Stasiewicz MJ, Wiedmann M (2013). Persistent *Listeria monocytogenes* subtypes isolated from a smoked fish processing facility included both phage susceptible and resistant isolates. Food Microbiol. 35:38-48.

Vorst KL, Todd EC, Rysert ET (2006). Transfer of *Listeria monocytogenes* during mechanical slicing of turkey breast, bologna, and salami. J. Food Prot. 69:619-626.

Zhang L, Yan Z, Ryser ET (2007). Impact of dilution ratios on *Listeria monocytogenes* growth during University of Vermont medium enrichment of deli meats. J. Food Prot. 70:2656-60.

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Full Length Research Paper

Safety characterization of *Yersinia enterocolitica* strains isolated from raw milk in Western Algeria

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***Yersinia enterocolitica*, an important food-borne enteric pathogen is associated with various clinical manifestations ranging from self-limited gastroenteritis to more invasive syndrome such as terminal ileitis and mesenteric lymphadenitis. The main aim of this study was to investigate the incidence of *Y. enterocolitica* in milk samples. For this purpose, one hundred (100) samples of raw cow's milk were collected from the western Algeria region. Seventeen (17) isolates were obtained. All these isolates belong to *Y. enterocolitica* biotype 1A and were *ystB* positive. Heat resistance and antibiotic susceptibility of these isolates were also investigated. The heat resistance D-value (decimal reduction time) and heat sensitivity Z_T values (increase in temperature leads to a ten-fold reduction of the D value) of *Y. enterocolitica* in BHI broth showed that D_{55}, D_{60} and D_{65} were 1.34, 0.85 and 0.62 min, respectively. The obtained Z value was 29.98°C and antibiotic resistance profiles of 17 isolates were evaluated. All the isolates were susceptible to 13 of the 30 tested antibiotic, resistance was noted for eight different antibiotics, among are them Ampicillin and 3rd generation Cephalosporins. The presence of chromosomal *ystB* gene virulence and antibiotic susceptibility indicate that these isolates from raw milk are potentially able to cause human foodborne illnesses and highlights the role of milk as a transmission vehicle of potentially pathogenic *Y. enterocolitica* strains, with consequent risks for consumer's health via the consumption of raw milk and derivatives.**

Key words: *Yersinia enterocolitica,* biotype, virulence gene, heat resistance, antibiotic resistance, raw milk.

INTRODUCTION

Yersinia enterocolitica, which was first described in 1934 as a small Gram-negative coccobacillus psychro-tolerant enterobacterium, isolated from several environmental sources, that is, foods and human clinical samples are a causative organism in several out-breaks of gastroenteritis, in which foods were implicated (Bottone, 1999; Soltan-Dallal et al., 2004; Lambertz and Danielsson-Tham, 2005). In recent years, *Y. enterocolitica* has been the third most common cause of food borne diseases after *Campylobacter* spp.

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and *Salmonella* spp. (EFSA, 2011).

Y. enterocolitica has six biotypes, biotypes 1B, 2, 3, 4, and 5 which are known to be pathogenic and those of biotype 1A are considered as nonpathogenic (Bottone, 1999; Soltan-Dallal et al., 2004). The biotype 1A strains are generally regarded as non-virulent. They lack pYV plasmid and major chromosomal virulence genes. Despite this, some biotype 1A strains produce disease symptoms indistinguishable from that produced by known pathogenic biotypes (1B, 2-5). Some biotype 1A strains are able to invade epithelial cells, resist macrophages and carry genes associated with virulence (Tennant et al., 2003; Bhagat and Virdi, 2010). The *ystB* gene is widely distributed in *Y. enterocolitica* biotype 1A strains where the production of *Yersinia* stable toxin Yst-b is the major contributor to diarrhea produced by biotype 1A strains (Singh and Virdi, 2004b).

The frequent association of *Y. enterocolitica* with raw milk (Bernardino-Varo et al., 2013) and the ability of this organism to grow in milk at refrigeration temperatures (Bari et al., 2011) have been well documented. Some *Y. enterocolitica* biotypes are considered as the major prevalent milk-borne pathogens (Bernardino-Varo et al., 2013); they are responsible for gastroenteritis and other syndromes in humans and animals (Huovinen et al., 2010; Singh and Virdi, 2004b). Thus, its control is important for the safety of refrigerated dairy products (Ye et al., 2014). *Y. enterocolitica* has been isolated from raw milk and pasteurized dairy products in several countries e.g. in the USA (Jayarao and Henning, 2001), China (Wang et al., 2010; Ye et al., 2014), Mexico (Bernardino-Varo et al., 2013), Brazil (Falcão et al., 2006), Iran (Soltan-Dallah et al., 2004; Rahimi et al., 2014; Jamali et al., 2015), India (Subha et al., 2009), Turkey (Guven et al., 2010), Nigeria (Okeke and Okwori, 2014), Egypt (Darwish et al., 2015) and other countries. In Algeria, the raw milk is still frequently consumed. National production of raw cow's milk is estimated at 2.3 billion liters. Only a third of this quantity is integrated to the industrial plants (ITELV, 2012), therefore, the most important issue about *Y. enterocolitica* is its control in raw milk and derivatives.

A broad spectrum of antibiotics has been widely used in agriculture to treat infections and improve growth and feed efficiency in livestock and poultry (Mathew et al., 2007). The need to use antibiotics in the treatment of humans and animals may lead to the development of mechanisms resistance antibiotic, causing a growing risk to human and animal health (Perkowska et al., 2011). For this reason, the use of antibiotic growth promoters in animal production must be prohibited or controlled in each country (Singh and Virdi, 2004a). Moreover, *Y. enterocolitica* produces beta-lactamase (penicillinase and cephalosporinase) that make them naturally resistant strains to Penicillins and Cephalosporins first and second generations (Singh and Virdi, 2004a). Systematic monitoring of the susceptibility of bacterial strains, including *Y. enterocolitica*, must therefore be regarded as highly justified to ensure appropriate treatment of humans and to limit the spread of microorganisms' drug resistance in animals (Perkowska et al., 2011).

MATERIALS AND METHODS

Sampling

One hundred (100) raw cow's milk samples were collected aseptically from cans and tanks at the level of the dock receipt of dairy plant "Giplait Mansourah" located in Tlemcen city (northwest of Algeria) Samples were taken in a 250 mL sterile container, then transported to the laboratory in ice boxes. The journey took 15 min. At the laboratory, samples were immediately processed.

Raw cow's milk collected at the dairy is from different areas of Western Algeria: Ain- Temouchent, Ain-Youcef Amieur, Beni Mester, Mansourah, Ouled Mimoun, Remchi, Sabra, Sebdou, Sidi Bel-Abbès and Tlemcen. Milk was brought by farmers in the early morning in cans and refrigerated tank trucks. The study covers the period from January to October 2013. On average, 2 to 3 samples per week were used. The number of farmers is about 700 with an average of 8 cows/ breeder.

Isolation and isolates identification

Y. enterocolitica strains were isolated using two enrichment steps, a pre-enrichment in Peptone Sorbitol Bile salts broth (PSB broth, Fluka, India) and an enrichment in Irgasan, Ticarcillin and potassium Chlorate broth (ITC broth, Fluka, India). One milliliter of each sample was added to 10 mL of PSB broth. The presumptive presence of *Y. enterocolitica* was checked after 4 weeks (28 days) of incubation at 4°C without shaking. One milliliter of each pre-enriched culture in PSB broth was added to 10 mL of ITC broth and incubated at 25°C for 48 h without shaking. In order to reduce the background contaminating flora, Aulisio's alkali treatment method was performed: 0.5 mL of each enriched ITC broth was treated with 4.5 mL of 0.5% KOH solution (prepared in 0.5% NaCl solution), stirred for 20 s (AFNOR, 2003). Then, a loopful of the mixture was streaked immediately on Mac Conkey agar (Fluka, India) and Cefsulodin, Irgasan Novobiocin agar (CIN agar, Fluka, India) and incubated for 24 to 48 h at 30°C (Figure 1). The presumptive isolates were exanimate by biochemical tests as described by the ISO 10273:2003 horizontal method for the detection of presumptive pathogenic *Y. enterocolitica* with the following tests: Gram staining, oxidase, catalase, indole production, tryptophane deaminase, glucose and lactose fermentation, gas formation from glucose, H_2S production, lysine decarboxylase, utilization of Simmons citrate, esculin hydrolysis, reduction of nitrate, mobility at 25 and 37°C and fermentation of xylose, mannitol and trehalose (Table 1) (AFNOR, 2003). The isolates were further identified by using the API 20E (BioMerieux, France). This system is still accepted as the good standard for the rapid identification of *Y. enterocolitica* (Tudor et al., 2008). The identification of biotype relies on a panel of biochemical tests as described in the ISO 10273-2003 method, allowing differentiation of pathogenic biotypes from the non-pathogenic biotype (AFNOR, 2003). The protocol of *Y. enterocolitica* isolation from raw milk and following identification and pathogenicity determination of the isolated strains is schematized in Figure 1.

Real-time PCR for detection of ystB gene

The PCR assays have been developed as an efficient tool for

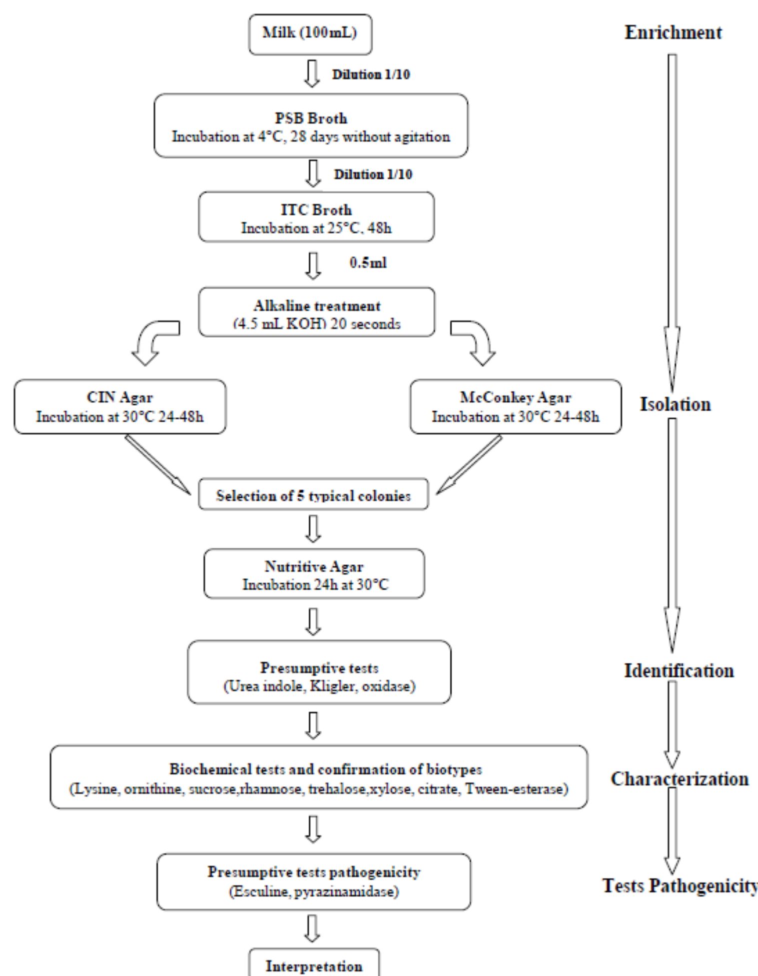

Figure 1. Schematic protocol of *Y. enterocolitica* isolation from raw milk and following identification and pathogenicity determination of the isolated strains.

Table 1. Some phenotypic and biochemical characteristics of *Y. enterocolitica* strains isolated from Algerian raw milk.

Test	Reaction	Test	Reaction
Gram staining	-	Oxidase	-
Utilization of Simmon's citrate	-	Catalase	+
Motility at 37°C	-	Gas production	-
Motility at 25°C	+	ONPG	+
Voges-Proskauer at 37°C	-	Lactose	-
Voges-Proskauer at 25°C	+	Sucrose	+
Lysine decarboxylase	-	Maltose	+
Ornithin decarboxylase	+	Mannitol	+
Urease activity	+	Rhamnose	-
Indole production	+	Arabinose	+
Nitrate reductase	+	Raffinose	-
H_2S production	-	Xylose	+
Kligler test	+	Salicin	-

(+): Positive reaction; (-): negative reaction.

identifying pathogenic *Y. enterocolitica* (Lambertz and Danielsson-Tham, 2005). Real time PCR targeted the chromosomally-located *ystB* gene that is present in all *Y. enterocolitica* strains (Wang et al., 2010). Strains were sub-cultured on (plate count agar) PCA at 30°C for 24 h. DNA was extracted from colonies with QIAamp DNA mini kit (Qiagen, USA) following the manufacturer's instructions. All PCR were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California), in a final volume of 25 µl with the Sybr® Green JumpstartTMTaqReadyMix TM (Sigma-Aldrich, Saint Louis, Missouri). The primers used in this study, were (5′-GTA CAT TAG GCC AAG AGA CG-3′) and (5′-GCA ACA TAC CTC ACA ACA CC-3′) (Baghat and Virdi, 2009). The final concentration of primers in the PCR reaction was 0.2 µM for *ystB*.

The PCR reaction was carried out under the following conditions: an initial denaturation at 95°C for 5 min, 34 cycles of 95°C for 10 s (denaturation), 55°C for 15 s (annealing) and 70°C for 1 min (extension) (Baghat and Virdi, 2009). The PCR products (146 bp) were visualized by ethidium bromide staining on 1.8% TBE agarose gel. Mass values are for 1 µg/lane. A 50- bp DNA ladder (Biolabs, New England) was used to determine the size of PCR product.

Heat treatment

One strain isolated from raw milk (baptized YHK261) was chosen for further characterization because of its unusual heat resistance. This strain was aseptically transferred to 100 mL Brain Heart Infusion Broth (BHI Conda, Spain) and incubated at 30°C for 24 h. 1 mL was sub-cultured in 100 mL BHI at 30°C for 18 h. At the stationary phase, cells were recovered and the culture was adjusted to a final colony count of 10^8 CFU mL^{-1}. Heating temperatures of 55, 60 and 65°C were chosen based on previous studies reported in literature (Pagán et al., 1999). The vials containing 100 mL of sterile BHI were placed in water-bath heated at the preselected temperature (e.g. 55°C). The vials are fixed so that the broth is totally submerged in the bath. The sample temperature during treatment was monitored using a thermometer (IsoLab GmbH, German) placed in another vial containing 100 mL of BHI simultaneously placed in the water bath with the first one, and to minimize any risk of contamination. In a first step, a bacterial suspension was introduced into the heating medium. Samples were removed periodically and immediately placed in ice-water. Each cooled sample was serially diluted in 9 mL of sterile physiological saline (0.9% NaCl w/v). During the third step, dilutions cascade was performed. Direct counts were obtained by plating in duplicate from the dilution series onto trypticase soy agar (TSA Conda, Spain). After incubation at 30°C for 24 h, plates were examined for typical colonies of *Y. enterocolitica*. The number of colony forming units (CFU) on agar plates was converted to $\log_{10}$ CFU g^{-1}. Each experiment was carried out in duplicate at each temperature.

The slope was obtained for each plot of $\log_{10}$ of surviving cells mL^{-1} against time using linear regression analysis Log_{10} D (T). The estimate of thermal resistance was obtained by fitting the linear regressions of the $\log_{10}$ number of surviving cells at each time interval. D values are the absolute value of the inverse slope of the regression line. These D values, in minutes, were used to fit plots of $\log_{10}$ D value versus temperature. To fit the models to the experimental data, the GraphPad PRISM (GraphPad Software, San Diego, CA, USA) was used. D values for *Y. enterocolitica* were calculated using the average slope for a given treatment. The value of the inverse slope obtained by plotting $\log_{10}$ D value versus temperature represents the Z value.

Antibiotic susceptibility testing

Y. enterocolitica isolates were examined for their susceptibility to b-lactam and non-b-lactam antibiotics. Antimicrobial susceptibility was determined by the standard disk diffusion method of Bauer, using Mueller-Hinton agar (Singh and Virdi, 2004a) and antibiotic disks were purchased from Pasteur Institute, Algeria. The plates were incubated (24 h at 37°C) and resistance was recorded via visual examination. Different antibiotics were tested (Table 2), including ampicillin (AM), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), oxacillin (OX), penicillin (P), ticarcillin/clavulanic acid (TCC), cefazolin (CZ), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), amikacin (AN), kanamycin (K), gentamicin (GM), tobramycin (TM), nalidixic acid (NA), ciprofloxacin (CIP), imipenem (IMP), trimethoprim/sulfamethozole (SXT), clindamycin (DA), colistin (CT), fusidic acid (FA), rifampin (RA), piperacillin (PI), aztreonam (ATM), chloramphenicol (C), streptomycin (S), tetracycline (TE), fosfomycin (FF) and erythromycin (E). Resistance to an antibiotic was confirmed using standard disk diffusion method. Break points to establish resistance were selected based on SFM recommendations for *Enterobacteriaceae* (Bonnet et al., 2010).

RESULTS AND DISCUSSION

Among one hundred analyzed raw cow milk samples, seven were contaminated by *Y. enterocolitica*. Seventeen isolates were identified as *Y. enterocolitica*. Contaminated milk are from five regions: Amieur, Mansourah, Ouled Mimoun, Sebdou and Tlemcen. The greatest number of positive samples was obtained from Ouled Mimoun farms (42.85% with n=7). All the isolates were biotyped by biochemical tests and detection of the virulence genes confirmed their biotype as 1A. The results concerning phenotypic and biochemical characterics of *Y. enterocolitica* strains isolated from west Algerian raw milk are summarized in Table 1.

The thermal death curves at the three treatment temperatures and the D values are shown in Figure 2. The corresponding D_{55}, D_{60} and D_{65} values were 1.34, 0.85 and 0.62 min, respectively. These values were determined experimentally and Figure 3 shows how Z-value was determined. Z value was strongly elevated: 29.98°C.

The raw milk contamination frequency by *Y. enterocolitica* obtained in this study (7%) was higher than that reported in other studies, 1.6% in Iran (Soltan-Dallal et al., 2004) and 1% in Nigeria (Okeke and Okwori, 2014). Therefore, the assessment of *Y. enterocolitica* virulence indicators does not need to be restricted to the detection of plasmid-localized genes of virulence, but requires, at least, one chromosomal virulence-associated gene to be present (Ye et al., 2014). The *ystB* gene is present in all strains of biotype 1A, similar results were also found by Platt-Samoraj et al. (2006) and Jamali et al. (2015). Some researchers believe that these strains *ystB*+ are pathogenic to humans and can cause local outbreaks (Singh and Virdi, 2004b, 2005). The study of Singh and Virdi (2004b) indicated that the *ystB* gene is widely distributed in *Y. enterocolitica* biotype 1A strains and production of *Yersinia* stable toxin Yst-b produced by biotype 1A strains is the major contributor to diarrhea. The presence of *ystB* gene was often associated with clinical cases and represents a risk that should not be ignored. Our results show that it is possible to detect

Table 2. Antibiotic susceptibility of the tested *Y. enterocolitica* strains isolated from Algerian raw milk.

Antibiotics	Disc charge (µg)	***Y. enterocolitica*** **strains (%) (n=17)**		
		S	**I**	**R**
Amikacin,	30	100	0	0
Amoxicillin	25	0	0	100
Amoxicillin/Clavulanic acid	30/10	0	0	100
Ampicillin	10	0	11.76	88.24
Aztreonam	30	88.24	11.76	0
Cefazoline	30	0	11.76	88.24
Cefotaxime	30	17.65	35.29	47.06
Cefoxitine	30	29.42	35.29	35.29
Ceftazidime	30	0	41.18	58.82
Ceftriaxone	30	0	5.88	94.12
Chloramphenicol	30	100	0	0
Ciprofloxacin	5	100	0	0
Clindamycin	2	0	0	100
Colistin	10	76.47	0	23.53
Erythromycin	15	0	0	100
Fosfomycin	50	100	0	0
Fusidic acid	10	0	0	100
Gentamicin	10	100	0	0
Impinem	10	100	0	0
Kanamycin,	30	100	0	0
Naldixic acid	30	100	0	0
Oxacillin	5	0	0	100
Penicillin	10	0	0	100
Piperacillin	30	100	0	0
Rifampine	30	17.65	76.47	5.88
Streptomycin	10	100	0	0
Tetracycline	30	100	0	0
Ticarcillin/Clavulanic acid	75/10	0	0	100
Tobramycin	10	100	0	0
Trimethoprim/Sulfamethozol	1.25/23.75	100	0	0

S= Susceptible, I=intermediate, R= resistant, n= number of tested strains.

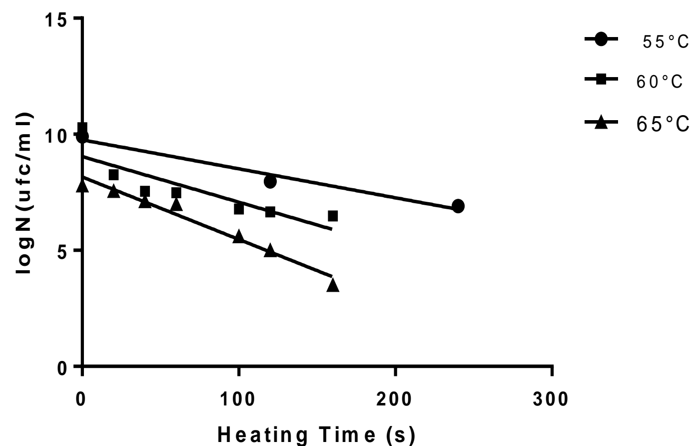

Figure 2. logN versus heating time at different heating temperature: 55°C ●, 60°C ■ and 65°C ▲ for *Y. enterocolitica* YHK261 strain (the experiment was run in duplicate).

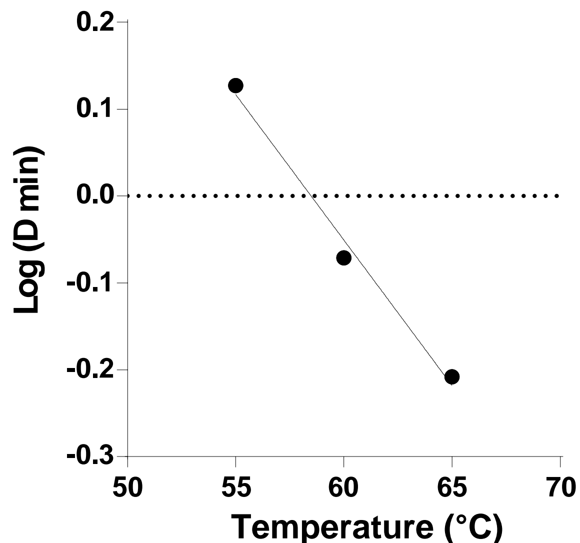

Figure 3. Temperature versus log for *Y. enterocolitica* YHK261 strain (the experiment was run in duplicate).

pathogenic strains with these traits.

Although, there is very little thermal inactivation data published in the scientific literature for *Y. enterocolitica*, Pagán et al. (1999) have reported D_{55} values of 0.33 to 0.78 min and D_{59} values of 0.18 to 0.6 min in citrate phosphate buffer. These values are less than those reported in this paper (1.34 and 0.85 min, respectively). A second study by Bolton et al. (2013) reported D_{55}, D_{60} and D_{65} values of 10.98, 2.53 and 0.60 min, respectively, it is noteworthy that the D_{65} is the same as that obtained. The discrepancies of values may be attributed to the strain variation (Bhagat and Virdi, 2009). This study suggests that minor changes in the temperature of the milk treatment will greatly influence the survival of *Y. enterocolitica* and that mild temperatures are sufficient for the elimination of this microorganism as compared to others such as *Mycobacterium avium subsp. paratuberculosis*, *Coxiella burnetii* and bacterial spores (Pearce et al., 2012). Augmenting the temperature from 55 to 60°C would reduce the D values 1.6 fold, a time-temperature combination of 0.85 min at 60°C is required to achieve one log reduction in *Y. enterocolitica*, the equivalent time at 65°C was 0.62 min.

This study provides D_T and Z values required to eliminate *Y. enterocolitica* and reduce the microbiological risk related to this microorganism without harming the organoleptic and nutritional quality of milk. Outbreaks caused by *Y. enterocolitica* strains have been reported after consumption of pasteurized milk (Ackers et al., 2000) however some heat resistance studies indicate that these strains are unable to survive to the pasteurization suggesting that their presence in pasteurized milk is either due to post-pasteurization contamination or under-processing (Greenwood et al., 1990). *Y. enterocolitica* can multiply at temperatures as low as 4°C (Bottone et al., 1999). Its presence in pasteurized milk, which is generally stored at refrigeration temperatures at the dairy, in the retail chain and at home, may have public health significance.

In Algeria, there is very little research related to food contamination by *Y. enterocolitica.* There are no reports of its presence in raw cow's milk, pasteurized milk, or milk derivatives. Notably, it is usual in this country to prepare dairy products from unpasteurized milk, although this food can be a vehicle of pathogens to humans. This work shows the potential of public health risk in Algeria regarding infections transmitted by raw cow's milk. Therefore, consumers are adviced to mild heat their milk before consumption to avoid the pathogenic *Y. enterocolitica* risk in raw milk or even in pasteurized milk.

Antibiotic susceptibility is of great importance. The results shown in Table 2 indicate that all the tested *Y. enterocolitica* strains (17/17) were susceptible to 13 antibiotics: Amikacin, Chloramphenicol, Ciprofloxacin, Gentamicin, Kanamycin, Naldixic acid, Streptomycin, Fosfomycin, Tetracyclin, Impinem, Piperacillin, Tobramycin and Trimethoprim/Sulfamethozol. Similar patterns of susceptibility were observed among strains isolated from pig tonsils in Switzerland, southern Germany as well as in human strains (Fredriksson-Ahomaa et al., 2009; Bucher et al., 2008). Piperacillin is a representative of ureidopenicillins with a broad scope of antibacterial activity. In the studies of Kot et al. (2008), 77.8% of *Y. enterocolitica* strains were found to belong to biotype 1A.

In veterinary medicine, treatment with antibiotics of penicillin group such as amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, oxacillin, amoxicillin, ampicillin and piperacillin is frequent. The literature cites assessments of *Y. enterocolitica* susceptibility to Amoxicillin, as well as Amoxicillin/Clavulanic acid. In this study, resistance to Ampicillin was shown in all the 17 isolates. Ampicillin showed strong bactericidal activity towards a wide range of microorganisms; however, *Y. enterocolitica* strains are in general resistant to this antibiotic. Ampicillin resistance due to production of β-lactamases is well described in the literature (Bucher et al., 2008). We found no susceptible strains and only 1.94% of strains to be intermediately susceptible to Ampicillin. Singh and Virdi (2004a) found 100% of strains to be resistant, which is also confirmed by results from other authors (Rastawicki et al., 2000). The combination of ampicillin with clavulanic acid significantly broadens the scope of activity and increases the percentage of susceptible strains. In the present study, all strains were fully resistant to amoxicillin/clavulanic acid and ticarcillin/clavulanic acid. The study of Singh and Virdi (2004a) demonstrated that only 2.5% of *Y. enterocolitica* strains were intermediately susceptible,

while the remaining strains were fully resistant to Amoxicillin/Clavulanic acid.

Fifteen strains of 17 (88.24%) are resistant to 1st generation Cephalosporins. This antibiotic group including Cefazolin, are active against Gram-positive and Gram-negative bacteria, while several studies proved that 90% of strains belonging to the *Enterobacteriaceae* family are resistant to Cefazolin (Fredriksson-Ahomaa et al., 2007; Bucher et al, 2008; Bhaduri et al., 2009). Kot et al. (2008) demonstrated that about 90% of biotype 1A *Y. enterocolitica* were susceptible to Cefazolin; these results are not in accordance with our data. Six (35.29%) strains are resistant to 2nd generation cephalosporins including cefoxitin and 6 (35.29%) strains are intermediately sensitive. The 2nd generation Cephalosporins have a stronger activity against Gram-negative than Gram-positive bacteria. Singh and Virdi (2004a) have demonstrated that 41.3 and 37.5%, of *Y. enterocolitica* strains are susceptible and 52.5 and 50% intermediately susceptible, respectively. The third generation Cephalosporins of which Ceftriaxone, Ceftazidime and Cefotaxime were included in earlier studies demonstrated bactericidal activity mainly against *Staphylococcus* sp. and *Streptococcus* sp., but also against *Enterobacteriaceae, Haemophilus influenzae, Borrelia* sp., and *Pasteurella* sp.

Y. enterocolitica are categorized as microorganisms susceptible to the 3rd generation Cephalosporins. In this study, 16, 10 and 8 (94.12, 58.82 and 47.06%) strains show resistance against Ceftriaxon, Ceftazidim and Cefotaxim, respectively. Singh and Virdi (2004a) did not find biotype 1A *Y. enterocolitica* strains resistant to the 3rd generation Cephalosporins. The studies of the Polish clinical strains of *Y. enterocolitica*, serotype O:3, have demonstrated full susceptibility of the strains to the 3rd generation Cephalosporins (Rastawicki et al., 2000). It may be concluded that in this study, the strains of *Y. enterocolitica* isolated from milk varied greatly in terms of their *in vitro* susceptibility to β-lactam antibiotics. *Y. enterocolitica* strains were found to be relatively highly resistant to Cephalosporins and most Penicillins. It is deemed necessary to systematically monitor the *Y. enterocolitica* strains susceptibility to antibiotics.

Due to *Y. enterocolitica* wide spread particularly in dairy product, inappropriate antibiotic treatment and prophylaxis, as well as antibiotics overuse in human and veterinary medicine, may lead to the development of resistant strains to one or several groups of antibiotics (Vose et al., 2001). Bacteria have wide mechanisms to develop antibiotic resistance, therefore it is important to systematically assess their susceptibility to individual antibiotics, thus enabling selection of an optimal treatment and preventing drug resistance spread among bacteria (Caprioli et al., 2000; Fredriksson-Ahomaa et al, 2009). The improper use of antibiotics in the developing countries like Algeria may be the main cause of high resistance rate in local *Yersinia* isolates.

Most importantly, preventive measures such as reasonable antibiotherapy must be adopted to avoid increasing resistance to antibiotics of *Y. enterocolitica*. Moreover using antibiotics as growth promoters must be prohibited.

Conclusion

This study provides data on the occurrence of *Y. enterocolitica* in raw milk in Western Algeria and their resistance ability to antibiotics. The risk due to the presence of *Y. enterocolitica* in raw milk is not insignificant. It highlights the role of raw milk and dairy derivatives as a transmission vehicle of potentially pathogenic *Y. enterocolitica* strains that can transmit antibiotic resistance to the intestinal flora. However, *Y. enterocolitica* is easily eliminated by heat treatment at mild temperatures of about 65°C.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ackers ML, Schoenfeld S, Markman J, Smith MG, Nicholson MA, DeWitt W, Slutsker L (2000). An outbreak of *Yersinia enterocolitica* O:8 infections associated with pasteurized milk. J. Infect. Dis. 181(5):1834-1837.

AFNOR (2003). Norme NF EN ISO 10273: Microbiologie des aliments : méthode horizontale pour la recherche de *Yersinia enterocolitica* présumées pathogènes. ISO 10273:2003.

Bari ML, Hossain MA, Isshiki K, Ukuku D (2011). Behavior of *Yersinia enterocolitica* in Foods. J. Pathog. 2011(13).

Bernardino-Varo L, Quiñones-Ramírez EI, Fernández FJ, Vázquez-Salinas C (2013). Prevalence of *Yersinia enterocolitica* in raw cow's milk collected from stables of Mexico City. J. Food. Prot. 76(4):694-698.

Bhaduri S, Wesley I, Richards H, Draughon A, Wallace M (2009). Clonality and antibiotic susceptibility of *Yersinia enterocolitica* isolated from US market weight hogs. Foodborne. Pathog. Dis. 6(3):351-356.

Bhagat N, Virdi JS (2010). The Enigma of *Yersinia enterocolitica* biotype 1A. Crit. Rev. Microbiol. 1-15.

Bhagat N, Virdi JS (2009). Molecular and biochemical characterization of urease and survival of *Yersinia enterocolitica* biotype 1A in acidic pH *in vitro*. BMC. Microbiol. 9(1):262.

Bolton DJ, Ivory C, McDowell D (2013). Thermal inactivation of *Yersinia enterocolitica* in pork slaughter plant scald tank water. Meat Sci. 95(3): 668-671.

Bonnet R, Cavallo JD, Chardon H, Chidiac C, Courvalin P, Drugeon H, Dubreuil L, Guery B (2010). Comité de l'Antibiogramme de la Société Française de Microbiologie. CA. SFM. (2010):19-40.

Bottone EJ (1999). *Yersinia enterocolitica*: overview and epidemiologic correlates. Microbes. Infect. 1(4):323-333.

Bucher M, Meyer C, Grötzbach B, Wacheck S, Stolle A, Fredriksson-Ahomaa M (2008). Epidemiological data on pathogenic *Yersinia enterocolitica* in Southern Germany during 2000-2006. Foodborne. Pathog. Dis. 5(3):273-280.

Caprioli A, Busani L, Martel JL, Helmuth R (2000). Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. Int. J. Antimicrob. Agents. 14(4):295-301.

Darwish SF, Asfour HA, Allam HA (2015). Incidence of *Yersinia*

enterocolitica and *Yersinia pseudotuberculosis* in raw milk samples of different animal species using conventional and molecular methods. A. J. V. S. 44(1):174-185.

European Food Safety Authority (EFSA) (2011). The community summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in the European Union in 2009. EFSA. J. 9(3):2090.

Falcão JP, Falcão DP, Pitondo-Silva A, Malaspina AC, Brocchi M (2006). Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. J. Med. Microbiol. 55(11):1539-1548.

Fredriksson-Ahomaa M, Stolle A, Stephan R (2007). Prevalence of pathogenic *Yersinia enterocolitica* in pigs slaughtered at a Swiss abattoir. Int. J. Food. Microbiol. 119(3):207-212.

Fredriksson-Ahomaa M, Wacheck S, Koenig M, Stolle A, Stephan, R (2009). Prevalence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in Switzerland. Int. J. Food. Microbiol. 135(3):199-202.

Greenwood MH, Hooper WL, Rodhouse JC (1990). The source of *Yersinia spp.* in pasteurized milk: an investigation at a dairy. Epidemiol. Infect. 104(03):351-360.

Guven A, Sezer C, Aydin BD, Oral NB, Vatansever L (2010). Incidence and pathogenicity of *Yersinia enterocolitica* isolates from foods in Turkey. Kafkas Univ Vet Fak Derg. 16:107-112.

Huovinen E, Sihvonen LM, Virtanen MJ, Haukka K, Siitonen A, Kuusi, M (2010). Symptoms and sources of *Yersinia enterocolitica*-infection: a case-control study. BMC. Infect. Dis. 10(1):122.

ITELV (Institut technique des élevages en Algérie) (2012). Info élevage, bulletin trimestriel n°6. 2012. http://www.itelv.dz/index.php/bulletins-agro-meteo/summary/3-infoselevages/21-infos-elevages-itelv-n6.html (consulted 29 April 2015).

Jamali H, Paydar M, Radmehr B, Ismail S (2015). Prevalence, characterization, and antimicrobial resistance of *Yersinia* species and *Yersinia enterocolitica* isolated from raw milk in farm bulk tanks. J. Dairy. Sci. 98(2):798-803.

Jayarao BM, Henning DR (2001). Prevalence of foodborne pathogens in bulk tank milk. J. Dairy. Sci. 84(10):2157-2162.

Kot B, Jakubczak A, Piechota M (2008). Isolation and characterization of *Yersinia enterocolitica* rods from pig tonsils. Med. Weter. 64(3):283-287.

Lambertz ST, Danielsson-Tham ML (2005). Identification and characterization of pathogenic *Yersinia enterocolitica* isolates by PCR and pulsed-field gel electrophoresis. Appl. Environ. Microb. 71(7):3674-3681.

Mathew AG, Cissell R, Liamthong S (2007). Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. Foodborne Pathog. Dis. 4(2):115-133.

Okeke OFI, Okwori AEJ (2014). Occurrence of pathogenic *Yersinia* species in locally fermented cow milk (nono) in jos Nigeria. N. J. B. 23.

Pagán R, Mañas P, Raso J, Trepat, FJS (1999). Heat resistance of *Yersinia enterocolitica* grown at different temperatures and heated in different media. Int. J. Food. Microbiol. 47(1):59-66.

Pearce LE, Smythe BW, Crawford RA, Oakley E, Hathaway SC, Shepherd, JM (2012). Pasteurization of milk: the heat inactivation kinetics of milk-borne dairy pathogens under commercial-type conditions of turbulent flow. J. Dairy. Sci. 95(1):20-35.

Perkowska K, Platt-Samoraj A, Bancerz-Kisiel A, Szweda W (2011). Susceptibility of Polish *Yersinia enterocolitica* strains isolated from pigs to 12 β- lactam antibiotics. Bull Vet Inst Pulawy. 55:397-402.

Platt-Samoraj A, Ugorski M, Szweda W, Szczerba-Turek A, Wojciech K, Procajło Z (2006). Analysis of the presence of *ail, ystA* and *ystB* genes in *Yersinia enterocolitica* strains isolated from aborting sows and aborted fetuses. J. Vet. Med. 53(7):341-346.

Rahimi E, Sepehri S, Dehkordi FS, Shaygan S, Momtaz H (2014). Prevalence of *Yersinia* Species in Traditional and Commercial Dairy Products in Isfahan Province, Iran. Jundishapur. J. Microbiol. 7(4).

Rastawicki W, Gierczyński R, Jagielski M, Kałużewski S, Jeljaszewicz J (2000). Susceptibility of Polish clinical strains of *Yersinia enterocolitica* serotype O3 to antibiotics. Int. J. Antimicrob. Agents. 13(4):297-300.

Singh I, Virdi JS (2004a). *In vitro* susceptibilities of *Yersinia enterocolitica* biotype 1A. World. J. Microbiol. Biotechnol. 20:329-331.

Singh I, Virdi JS (2004b). Production of *Yersinia* stable toxin (YST) and distribution of *yst* genes in biotype 1A strains of *Yersinia enterocolitica*. Japan. J. Infect. Dis. 53(11):1065-1068.

Singh I, Virdi JS (2005). Interaction of *Yersinia enterocolitica* biotype 1A strains of diverse origin with cultured cells in vitro. Jpn. J. Infect. Dis. 58(1):31-33.

Soltan-Dallal MM, Tabarraie A, Moez Ardalan K (2004). Comparison of four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk from Northern Iran. Int. J. Food. Microbiol. 94(1):87-91.

Subha B, Ramakrishnan D, Suganthi V (2009). Antimicrobial resistance pattern of selected *Yersinia enterocolitica* isolated from raw cow milk and pork samples of Namakkal District, Tamilnadu, South India. I. J. E. R. 3(3):169-177.

Tennant SM, Grant TH, Robins-Browne RM (2003). Pathogenicity of *Yersinia enterocolitica* biotype 1A. FEMS. Immunol. Med. Microbiol. 38(2):127-137.

Tudor L, Ţogoe I, Ceauşi C, Mitrănescu E, Goncearov M, Rotaru E (2008). The optimization of *Yersinia enterocolitica* isolation and identification from food products. Lucrari. Stiintifice. USAMV.41:993-1002.

Vose D, Acar J, Anthony F, Franklin A, Gupta R, Nicholls T, Tamura Y, Thompson S, Threlfall EJ, Van Vuuren M, White DG, Costarrica ML (2001). Antimicrobial resistance: risk analysis methodology for the potential impact on public health of antimicrobial resistant bacteria of animal origin. Rev. Sci. Tech. OIE. 20(3):811-827.

Wang X, Cui Z, Wang H, Tang L, Yang J, Gu L, Jin D, Luo L, Qiu H, Xiao Y, Xiong H (2010). Pathogenic strains of *Yersinia enterocolitica* isolated from domestic dogs (Canisfamiliaris) belonging to farmers are of the same subtype as pathogenic *Y. enterocolitica* strains isolated from humans and may be a source of human infection in Jiangsu Province, China. J. Clin. Microbiol. 48(5):1604-1610.

Ye YW, Ling N, Han YJ, Wu QP. (2014). Detection and prevalence of pathogenic *Yersinia enterocolitica* in refrigerated and frozen dairy products by duplex PCR and dot hybridization targeting the *virF* and *ail* genes. J. Dairy. Sci. 97(11): 6785-6791.

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Full Length Research Paper

Isolation, characterization, and properties study of probiotic lactic acid bacteria of selected yoghurt from Bangladesh

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Probiotics, as functional food components, are recognized as safe microorganisms of viable single or mixed cultures with claimed health promoting effects on their host by improving the properties of the indigenous intestinal microflora. In the present study, a total of ten probiotic lactic acid bacteria were isolated comprising *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Bifidobacterium* sp. and *Streptococcus thermophilus*. According to morphological, physiological, and biochemical assays, all the isolates were gram positive, endospore negative, catalase negative, non-motile, and possessed bile salt hydrolase activity characteristic to probiotic bacteria. Carbohydrate fermentation profiles ensured the presumptive identification. Importantly, the isolates were resistant to artificial gastric juice environment at pH 2.2, and their resistance decreased after 24 h of incubation at 37°C. Moreover, bile salt tolerance was observed not only at 0.05, 0.1, 0.15, and 0.3% artificial bile from 0 to 4 h of incubation at 37°C, but also started multiplication after 16 h. The best phenol tolerance found at 0.1 to 0.2% phenol, very low at 0.3 and 0.4% phenol after 12 and 24 h of incubation, respectively. They also possessed excellent tolerance against 1 to 7% NaCl. Because of being probiotic potentiality, the best isolates can be used for probiotic product development in future.

Key words: Probiotics, artificial bile, artificial gastric juice, bile salt hydrolase, carbohydrate fermentation.

INTRODUCTION

The word ‘probiotic’ derived from the Greek word ‘pro bios’ which means ‘for life’. According to World Health Organization (WHO)/Food and Agriculture Organization (FAO), probiotics are ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’. According to Salminen et al. (1998), probiotics can be defined as ‘a live microbial food ingredient that is beneficial to health’.

Lactic acid bacteria are ubiquitous in nature and their nutritional requirements are highly complex. Therefore, their predominate habitats are rich in carbohydrates, protein breakdown products, vitamins, and environments with low level of oxygen. This confirms the prevalence in various kinds of dairy products (Stiles and Holzapfel,

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1997). Most probiotic microorganisms belong to the group of lactic acid bacteria (LAB) that reportedly play a significant role in maintaining the intestinal ecosystem and in stimulating the immune system of the host (Saarela et al., 2002). Species of the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics of human food and animal feeds (Belicova et al., 2013). The strains of *Lactobacillus, Bifidobacterium,* and *Streptococcus* have traditionally been used in the manufacture of various kinds of fermented dairy products and are generally regarded as safe (GRAS status) according to the American Food and Drug Administration due to their long history of safe use in fermented foods and feeds, and their presence in the intestinal and urogenital microbiota (O'Sullivan et al., 1992; Belicova et al., 2013). In addition, these bacteria are desirable members of the intestinal microflora (Berg, 1998). A clinical study revealed that, *Lactobacillus* species containing probiotics are associated with a reduction in antibiotic associated diarrhea and boost the immune system (Hempel et al., 2012). Lack of pathogenicity, tolerance to gastrointestinal conditions, such as gastric acid and bile, tolerance to phenol, NaCl, bile salt hydrolase activity are some of the general criteria for the selection of probiotics (Collins et al., 1998; Ouwehand et al., 2002a; Pereira et al., 2003; Hoque et al., 2010). LAB may provide beneficial health effects by modifying the host immune system by reducing the colonization of pathogenic microorganisms and promoting healing of damaged mucosa during bacterial adhesion to the epithelium (Ouwehand et al., 2002b).

Lactobacillus acidophilus and *Bifidobacterium bifidum* were used to make mildly acidified yogurts called "bio-yogurts" in Germany during late 1960s (Goktepe et al., 2006). Viable probiotic strains with beneficial functional properties are supplied in the markets as fermented food products, mainly "yogurt"-type, or in lyophilized form, both as food supplements and as pharmaceutical preparations. For many years, pharmaceutical preparations contain live microorganisms in capsules, also known as "biotherapeutics" after or during antibiotic treatment (Goktepe et al., 2006). As probiotic bacteria have potential therapeutic or prophylactic effects, so development of numerous probiotic products such as fermented milk drinks, yoghurt, cheese, ice-cream, sausages etc. with defined starter culture are quite demanding in the markets. However, industrial applications of probiotics are quite challenging.

Yoghurt is regarded as one of the most popular probiotic food worldwide with claimed health benefits, ranging from high nutrition value, reduction of duration of diarrhea, reduction of blood cholesterol, obesity, gastrointestinal disorders, diabetes, overall stimulation of immune system, control of gastrointestinal pathogens through antimicrobial compounds, improve lactose digestion in lactose intolerant individuals, etc. Yoghurt is defined by the *Codex Alimentarius* of 1992, the result of coagulation of milk, causing the lactic acid of *Streptococcus thermophilus*, *Bifidobacterium* species, *Lactobacillus bulgaricus* and other species depending on regional differences (Bourlioux and Pochart, 1988). Yogurt gels are built of clusters of aggregated case in particles formed as a result of gradual fermentation of lactose by lactic acid bacteria (Horne, 1999). According to the National Yoghurt Association, the criteria for active and live yoghurt is the finished yoghurt containing live lactic acid bacterial count of $\geq 10^8$ CFU/g at the time of production, and cultures must live and be active at the end of defined self-life (Chandan and Shahani, 1993). Consumption of milk and milk products was traced back to the time when people used domesticated mammals. It is commonly decided among historians that yogurt and other fermented milk products were discovered by chance as a consequence of milk being stored in warm climates. Most historical accounts attribute yogurt to the Neolithic peoples of Central Asia around 6000 B.C. Herdsmen began the practice of milking their animals and the natural enzymes in the carrying containers (animal stomachs) curdled the milk, essentially making yogurt. Not only did the milk then keep longer, it is thought that people preferred the taste, so they continued the practice, which then evolved over centuries into commercial yogurt making. Yoghurt was thought to be originated from the Middle-East. The word 'yoghurt' is derived from the Turkish word, 'yoğurt', which means "to curdle or coagulated; to thicken". The first written description of yoghurt was printed in Diwanu I-Lugat al-Turk, a Turkish dictionary written in 100 to 1073. The popularity of yogurt soared in the 50 and 60s with the boom of the health food culture and is now available in many varieties to suit every taste and lifestyle (DNR, 2014). Yogurt is also very healthy as a part of daily diet. The most common types of yogurt are set type yogurt and strained yogurt. Set type yogurt is fermented in containers and no water removal takes pace after the fermentation. Strained or Greek style yogurt is fermented in tanks under continuous mild stirring and after the completion of fermentation, a portion of the whey is removed.

Yogurt is mainly produced from bovine milk. Raw milk undergoes centrifugal clarification to remove somatic cells and solid impurities, followed by a mild heating process, known as thermalization, at 60 to 69°C for 20 to 30 s. The purpose of heat treatment is to kill any vegetative microorganisms and the partial inactivation of unwanted enzymes. Then, milk is cooled at <5°C, at that time, inoculation with lactic acid bacteria is performed for fermentation of milk lactose to produce lactic acid by the action of enzyme lactase. Lactase converts lactose into glucose and galactose, which upon glycolysis and fermentation produce lactic acid at the end. The fat content of the milk is adjusted to range from <0.5% for skim milk to 1.5 to 2% for semi-fat milk to 3.5% for full fat milk. The fat content ranges from 0.1 to 10% according to

consumer demands. Fermentation process is the starter culture that acts through biochemical reactions and inductively causes the formation of the curd and the development of flavor components when incubated overnight at 37 to ~40°C. After incubation, the fermentation period is completed by lowering the temperature to 4°C and the produced yoghurt is ready for package and commercial distribution and consumption (Sfakianakis and Tzia, 2014). It is a good source of calcium, phosphorus, riboflavin-vitamin B2, iodine, vitamin B12, pantothenic acid-vitamin B5, zinc, potassium, protein, and molybdenum. Yogurt is also high in probiotics that can help a person live longer. The bacteria can also help boost the immune system. People who have allergies to dairy products are advised to consume yogurt as it does not produce the allergy that is caused by lactose. Yogurt is also a good option for people who suffer from stomach ailments, such as diarrhea. Consumption of low-fat yogurt can also aid in weight loss. Yoghurts are distributed and consumed in three different ways depending on regional preferences. Firstly, the Balkan style or set style yoghurt with a thick texture, consumed in the Middle-East and India. Secondly, Swiss style or stirred style yoghurt, slightly thinner than Balkan-style or set yogurt can be eaten as-is, in cold beverages or incorporated into desserts or fruits. Thirdly, Greek style yoghurt, which is a very thick yogurt that is either made from milk that has had some of the water removed or by straining whey from plain yogurt to make it thicker and creamier. Yoghurts are now industrially produced and commercialized into different shapes. These are frozen type yoghurt, yoghurt drink, fat free, gluten free, artisanal type, organic type, fruits mixed yoghurt, etc.

However, Bogra district in Bangladesh is famous for the finest quality of artisanal yoghurt production in terms of taste and odor. Therefore, to accomplish part of the probiotic product development steps, such as isolation of probiotic bacteria, characterization and probiotic properties determination of isolated bacteria, selective Bogra district yoghurts of Bangladesh were considered in this study. Moreover, to the best of our knowledge, literature review revealed that no such extensive studies regarding regional yoghurt was conducted so far in Bangladesh.

Therefore, the present research work was undertaken with the following objectives:

(1) Isolation and presumptive characterization of probiotic lactic acid bacteria from selected yoghurt.
(2) Study of probiotic properties of identified probiotic lactic acid bacteria.

MATERIALS AND METHODS

Collection of samples

Four artisanal yoghurt samples were collected from Bogra district of Bangladesh. The experiments were conducted at the “Food and Molecular Biotechnology Laboratory” of “Biotechnology and Genetic Engineering Discipline”, Khulna University, Bangladesh. For collection, the newly manufactured samples were transported in ice and stored for several hours aseptically at 4°C in refrigerator to protect from deterioration and contamination. Pour-plate method was conducted immediately at the same day for colony morphology observation and isolation. The famous shops from which the samples were collected were:

1. Sample No. 01: Ruchita Hotel and Restaurant, Sheikh Sorifuddin Super Market, Bogra.
2. Sample No. 02: Gourogopal Dodhi and Mistanno Vhandar, Nabab Bari More, Bogra.
3. Sample No. 03: Mohorom Ali Dahi Ghor, Station Road, Satmatha, Bogra.
4. Sample No. 04: Sherpur Dahi Ghor, Station Road, Satmatha, Bogra.

Chemicals and equipments

All the chemicals, sugars/carbohydrates, MRS, and ST culture media, motility–Indole-Lysine (MIL) medium components, and staining kits were purchased from the Sigma-Aldrich (India). 3% H_2O_2 (Sigma-Aldrich, USA) was purchased for catalase test. Bench top centrifuge (model 5415D, Eppendorf, Hamburg, Germany) was used for centrifugation purposes, and microplate reader (Multiskan FC Microplate photometer, USA) was used for optical density measurements.

Isolation of probiotic bacteria

For each sample, 1 g of yoghurt was dissolved in 9 ml of sterile peptone water solution (0.15% peptone), and serially diluted up to ten logarithmic (10^{-10}) fold. The diluted sample was then inoculated into the De-Man Rogosa Sharpe (MRS) agar plate for *Lactobacillus* species isolation or ST agar plate for *Streptococcus* species isolation by ensuring the optimum pH, incubation temperature, and incubation time anaerobically as shown in Table 1. The isolated cultures were maintained in MRS broth as a pure culture at pH 6.5 (International Dairy Federation, 1998). *Lactobacillus* spp. was isolated from the collected yoghurt using MRS media (De Man et al., 1960). *Bifidobacterium* spp., *S. thermophilus*, *L. acidophilus,* and *Lactobacillus brevis* are the most widely used species for commercial production of yoghurt and hence were targeted for isolation in the present study (Martini et al., 1991). For isolation, *L. acidophilus* was subjected to cultivate on MRS agar medium supplemented with 0.5% salicin (Dave and Shah, 1996), while L-cysteine (0.05%) was added to MRS medium to improve the specificity for isolation of *Bifidobacterium* spp. (Zinedine and Faid, 2007). ST agar medium was used for isolation of *S. thermophilus* (Driessen et al., 1977).

Lactic acid bacteria characterization

Isolates were further purified by streaking repeatedly and colony morphologies were observed. *S. thermophilus* colonies were selected based on coccoid shape, spherical or oval and occur in chains from samples No. 01 and 03. In addition, rod shaped, regular in long chains were selected for isolation of *L. acidophilus* from samples No. 01, 02, and 04. Rod shape, regular, occur singly/chain were observed for *L. brevis* from sample No. 01, 02, 03, and 04. Tiny rod, branched, v and y arrangement in chains were observed in *Bifidocterium* spp. from sample No. 02 (Table 2). Gram staining and catalase test were performed according to standard

Table 1. Optimum conditions for growth of lactic acid bacterial (LAB) isolates (Linn et al., 2008; Hoque et al., 2010; Saccaro et al., 2011)

Species name	Medium pH	Incubation time (h)	Incubation temperature (°C)
Lactobacillus acidophilus	6.5	24	37
Bifidobacterium spp.	5.2	72	45
Lactobacillus brevis	6.5	24	37
Streptococcus thermophilus	6.8	48	37

Table 2. Relation among sample collection shop, sample number and isolate number.

Shop name	Sample number	Isolate number	Species name
Ruchita Hotel and Restaurant	01	01	*Streptococcus thermophilus*
Mohorom Ali Dahi Ghor	03	02	*Streptococcus thermophilus*
Sherpur Dahi Ghor	04	03	*Lactobacillus brevis*
Gourogopal Dodhi and Mistanno Vhandar	02	04	*Bifidobacterium* spp.
Gourogopal Dodhi and Mistanno Vhandar	02	05	*Lactobacillus brevis*
Gourogopal Dodhi and Mistanno Vhandar	02	06	*Lactobacillus acidophilus*
Ruchita Hotel and Restaurant	01	07	*Lactobacillus acidophilus*
Mohorom Ali Dahi Ghor	03	08	*Lactobacillus brevis*
Sherpur Dahi Ghor	04	09	*Lactobacillus acidophilus*
Ruchita Hotel and Restaurant	01	10	*Lactobacillus brevis*

procedures. Schaeffer and Fulton (1933) method was employed for staining endospores.

Carbohydrate/Sugar fermentation profiles were done according to Erkus (2007) and Rahman et al. (2015), using sixteen different carbohydrates. Briefly, the first step was the preparation of active cells free from sugar residues (centrifugation at 10,000 rpm for 10 min, followed by resuspending the pelleted cells in 10 ml MRS without glucose, and containing bromecresol purple). The second step was the preparation of sterile sugar solutions. Finally, sugar solutions and active cell culture without sugar were combined. 200 µl of active cell solution without sugar was used as negative control. After overnight incubation at 37°C, the turbidity and the color change from purple to yellow with respect to negative and positive controls were recorded as positive fermentation result. All the reactions were performed in triplicate using 96-well microtiter plates.

Motility-Indole-Lysine (MIL) medium was used to determine the motility of microorganisms (Difco, 1998; Reller and Mirrett, 1975). Using a sterile needle, a well-isolated colony was picked and the medium was stabbed within 1 cm of the bottom of the tube. Incubation was accomplished at 35°C for 18 h or until growth was evident. A positive motility test result was indicated by a diffuse cloud of growth away from the line of inoculation. For maintenance, subcultures were prepared every week for maintenance of lactic acid bacteria for daily or weekly use.

Study of probiotic properties of lactic acid bacteria

Gastric juice resistance and bile salt tolerance were assayed using the method of Graciela and Maria (2001) with some modification and Zinedine and Faid (2007) to some extent where it was necessary. OD_{620nm} of cell growth in gastric juice and bile salt medium were taken for detecting the cell resistance and multiplication using microtiter plate reader. At 0 h, 1% of overnight bacterial culture was inoculated to the culture medium containing gastric juice (pH 2.2), or bile salt.

MRS broth was modified with 0.1 to 0.4% phenol to determine the phenol tolerance of the isolates. At 0 h, 1% of overnight bacterial culture inoculated to the culture medium containing 0.1 to 0.4% phenol. Uninoculated phenol solution served as negative control. Different concentrations (1 to 10%) of NaCl were inoculated into MRS medium. Growths were observed based on turbidity (Hoque et al., 2010). MRS agar medium with 0.5% (w/v) of the sodium salt of taurodeoxycholic acid (TDCA) was used to prepare test plates for plate assay (Dashkevicz and Feighner, 1989).

RESULTS

All the isolates were gram positive due to retain violet blue color and catalase negative due to the absence of catalase enzyme, confirmed by the lack of production of H_2O and O_2, when colonies were treated with H_2O_2. Moreover, endospore staining is one tool in the arsenal of bacterial identification method. Bacterial endospores are differentiated cells formed within the vegetative cells. The observed vegetative cells of the isolates were brownish red to pink, and no indication of bright green endospore, and hence all were endospore negative. In addition, motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms. The isolates were observed to be non-motile, indicated by growth along the inoculation line, but not further. Physiological and biochemical characteristics are shown in Table 3. Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific

Table 3. Physiological and biochemical characteristics of isolated LAB.

Physiological and biochemical characteristics	***Streptococcus thermophilus*** **(Isolate No. 01 and 02)**	***L. acidophilus*** **(Isolate No. 06 07, and 09)**	***L. brevis*** **(Isolate No. 03, 05, 08, and 10)**	***Bifidobacterium*** **spp. (Isolate No. 04)**
Gram staining	+	+	+	+
Catalase	-	-	-	-
Endospore	-	-	-	-
Motility	-	-	-	-
Bile salt hydrolase	+	+	+	+

Table 4. Presumptive identification of isolated LAB by sugar/carbohydrate fermentation pattern.

Isolate number	Lactose	Mannitol	Sucrose	Fructose	Salicin	Ribose	Cellubiose	Glucose	Maltose	Xylose	Rhamnose	L-Arabinose	D-Sorbitol	D-Mannose	Raffinose	Galactose	Conclusion/Presumptive identification
Isolate No. 01	++	--	++	++	++	++	++	++	++	+	--	++	++	++	--	++	*Streptococcus thermophilus*
Isolate No. 02	++	--	++	++	++	+	++	++	++	+	--	++	++	++	--	++	*Streptococcus thermophilus*
Isolate No. 03	++	+	++	++	++	++	++	++	++	++	--	++	--	++	-	++	*Lactobacillus brevis*
Isolate No. 04	++	+	++	++	++	++	++	++	++	++	--	+	--	++	+	++	*Bifidobacterium* spp.
Isolate No. 05	++	+	++	++	++	++	++	++	++	++	--	+	--	++	+/-	++	*Lactobacillus brevis*
Isolate No. 06	++	+/-	++	++	++	++	+/-	++	++	++	++	++	++	++	--	++	*Lactobacillus acidophilus*
Isolate No. 07	++	--	++	++	++	++	++	++	++	++	--	++	--	++	--	++	*Lactobacillus acidophilus*
Isolate No. 08	++	+	++	++	++	++	++	++	++	++	--	++	--	++	--	+	*Lactobacillus brevis*
Isolate No. 09	++	--	++	++	++	+	++	++	++	++	--	++	--	++	--	+	*Lactobacillus acidophilus*
Isolate No. 10	++	+/-	++	++	++	++	++	++	++	++	--	++	--	+	+	+	*Lactobacillus brevis*

(++) Excellent fermentation; (+) moderate fermentation; (- -) no fermentation, (-) very low fermentation, (+/-) low fermentation. LAB species identification results in Table 4 were analyzed based on Erkus (2007), Tamime (1985), Karna et al. (2007), Erdogrul and Erbilir (2006) and Rahman et al. (2015).

carbohydrate. Fermentation patterns can be used to differentiate among bacterial groups or species (Bartelt, 2000; Forbes et al., 2007; MacFaddin, 2000). Therefore, a total of 16 carbohydrates were used to presumptively identify the species of the isolates. The change of purple color of the MRS broth medium to yellow was the indication of fermentation due to lactic acid production (Table 4).

The isolates had the ability to survive in artificial gastric acid environment at low pH (pH 2.2), but their survival ability decreased after 24 h of incubation at 37°C (Figure 1). In addition, isolates 1 and 2 of the *S. thermophilus* showed the best tolerance in the gastric juice environment after 1 and 2 h of incubation in comparison to most of the isolates. Interestingly, the isolates exhibited reduced tolerance at pH 2.2, after 3 h of incubation

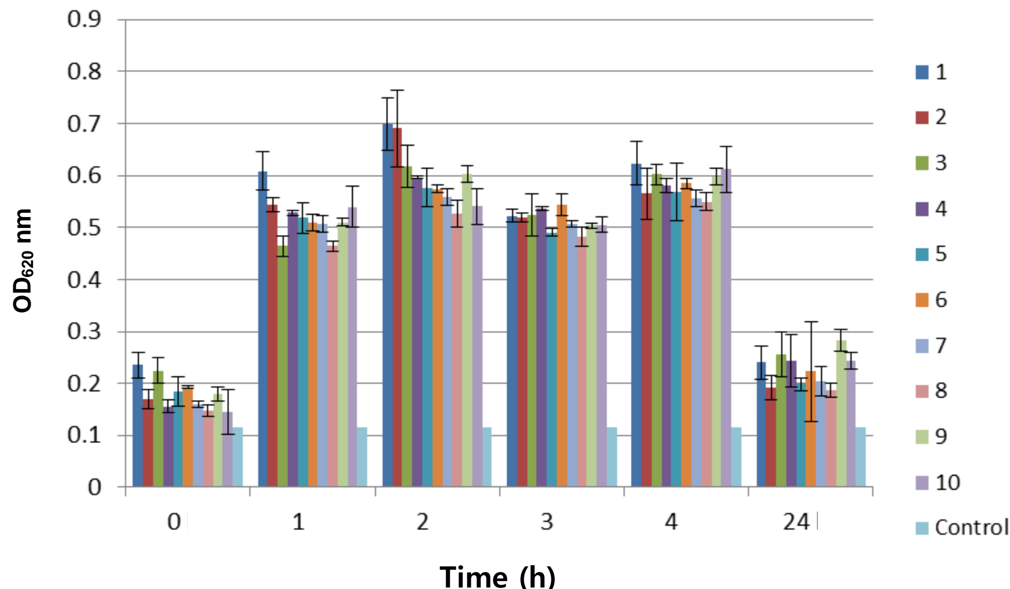

Figure 1. Survival and multiplication abilities of identified lactic acid bacterial isolates in artificial gastric juice at pH 2.2. Uninoculated medium was served as negative control. High $OD_{620\ nm}$ value bar diagram lines indicate more gastric juice resistance ability. Bars indicated standard error of the averages (n=3).

as compared to 2 h and further multiplication observed after 4 h of incubation (Figure 1). Furthermore, tolerance to 0.05, 0.1, 0.15, and 0.3% bile salt exhibited after 16 and 24 h of incubation at 37°C (Figure 2). From 0 to 4 h, the isolates were not started multiplication and as the times elapsed, they were able to grow in the bile salt environment. Importantly, after 24 h, their growth was the highest and indicated a sign of probiotic potentiality of all the isolates as excellent bile salt tolerance capability.

After 24 h, excellent tolerance and growth was detected at 0.1 and 0.2%, but very low at 0.3 and 0.4% phenol (Figure 3). Moreover, excellent NaCl tolerance was detected at 1 to 7% NaCl, moderate at 8 and 9%, but no growth occurred at 10% by observing turbidity (Table 5). At 0.1 to 0.3% phenol, isolate No. 07 showed the best tolerance ability as compared to other three isolates of *L. acidophilus*, but at 0.4% phenol, isolate No. 06 showed the best tolerance ability after 12 and 24 h of incubation (Figure 3). A 0.4% concentration of phenol causes a bacteriostatic action in some microorganisms (Xanthopoulos et al., 2000). When bile salt hydrolase-producing isolated and identified lactic acid bacteria were streaked out on MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA), the taurine-conjugated bile acid was deconjugated, producing deoxycholic acid. This deconjugation activity of isolates colonies were turn into opaque granular white colonies or precipitate halos around colonies characteristic to bile salt hydrolase-activity. Copius amount of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium or producing precipitate halos. The production of opaque granular white colonies or precipitate halos around colonies indicated bile salt hydrolase-active of all the isolates of the present study showing one of the most important characteristics of probiotic bacteria. A plus (+) sign indicates white granular opaque colonies (Table 3).

DISCUSSION

Recent understanding of the functions of intestinal microflora and the use of probiotic microorganisms is a novel concept to improve human health and an innovative approach for new food product development in functional foods for specific diseases. In this study, efforts were made to isolate and identify the best yoghurt lactic acid bacteria from the best artisan yoghurt production district Bogra in Bangladesh. For this purpose four different samples of yogurt were selected finally from the best shops. A total of ten lactic acid bacteria were isolated and identified and their probiotic properties were evaluated to determine which species will be the best choice for future probiotic product development attempt. The main task of carbohydrate fermentation test is to investigate the ability of bacteria to ferment different types of carbohydrate and uses as a method to identify the species. Therefore, species identification of the present study was determined using sixteen carbohydrates as the main species identification assay method. Ability to ferment carbohydrates of a particular LAB species is not exactly the same, because of geographical differences of the country, regional location

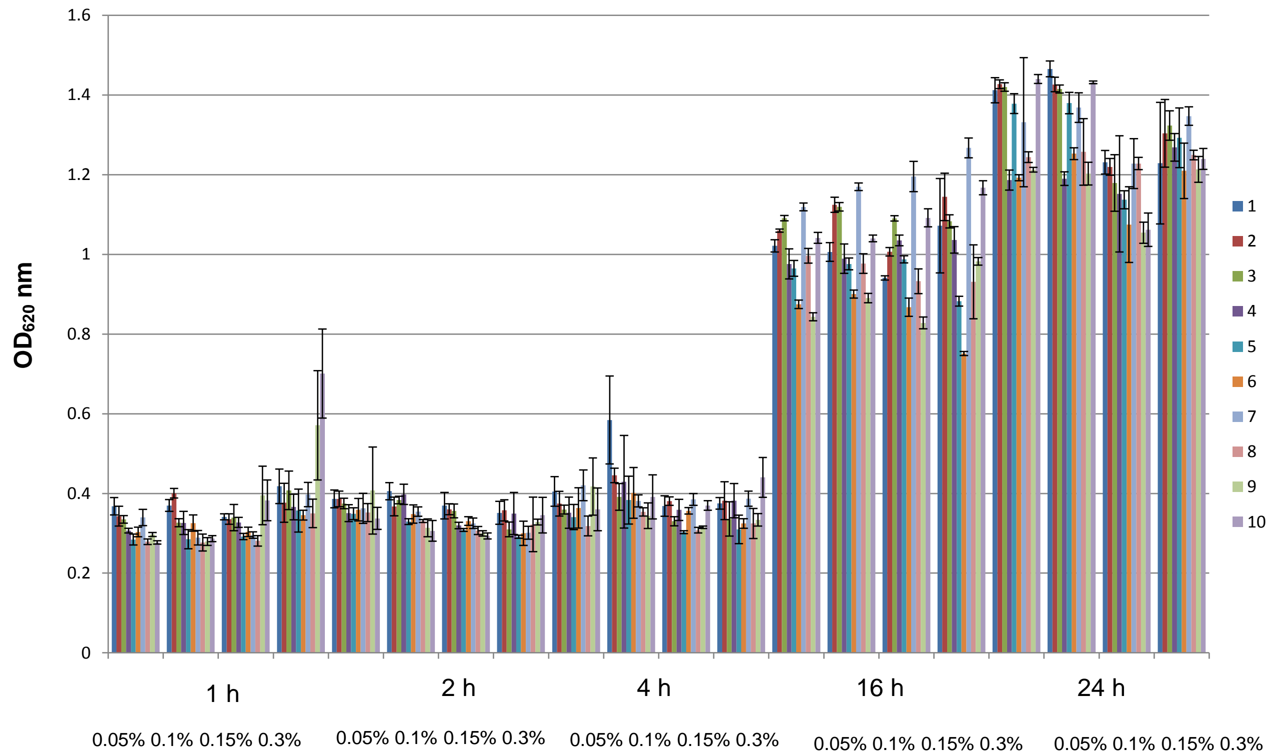

Figure 2. Bile salt tolerance of the isolates at 0.05, 0.1, 0.15, and 3% concentrations at 1, 2, 4, and 24 h of incubation at 37°C. Uninoculated bile salt medium was served as negative control ($OD_{620\ nm}$ 0.21). High $OD_{620\ nm}$ value bar diagram lines indicate more bile salt tolerance ability. Bars indicated standard error of the averages (n=3).

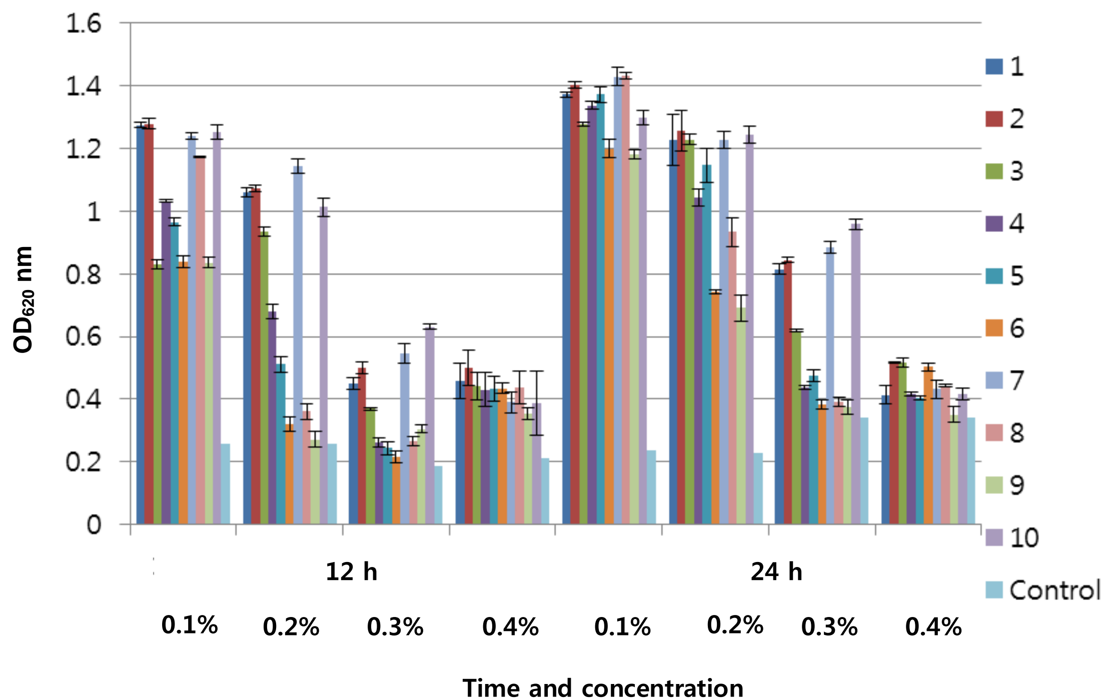

Figure 3. Tolerance of the isolates at 0.1, 0.2, 0.3, and 0.4% phenol after 12 h and 24 h of incubation at 37°C. High $OD_{620\ nm}$ value bar diagram lines indicate more tolerance ability. Uninoculated phenol medium was used as negative control. Bars indicated standard error of the averages (n=3).

Table 5. NaCl tolerance test of isolated LAB.

NaCl concentration (%)	Isolate No. 1 (Sample No. 01)	Isolate No. 2 (Sample No. 03)	Isolate No. 3 (Sample No. 04)	Isolate No. 4 (Sample No. 02)	Isolate No. 5 (Sample No. 02)	Isolate No. 6 (Sample No. 02)	Isolate No. 7 (Sample No. 01)	Isolate No. 8 (Sample No. 03)	Isolate no. 9 (Sample No. 04)	Isolate No. 10 (Sample No. 01)
1	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++
7	++	++	+	++	++	++	++	++	++	++
8	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	-

(++) Indicates excellent growth; (+) indicates moderate growth; (-) indicates no growth.

within the country, method of preparation, preservation of the yoghurt sample, etc.

According to the derived data (Figures 1, 2, and 3) on the resistance to artificial gastric juice at pH 2.2, bile salt and phenol tolerance, it was revealed that isolate No. 01 of *S. thermophilus* was better than isolate No. 02, while isolate No. 10 was the best among the four isolates of *L. brevis*, and all the three *L. acidophilus* isolates designated isolate No. 06, 07, and 09 were equally potent. The results of the gastric juice resistance were found similar to the results of Rahman et al. (2015). In addition, Hoque et al. (2010) isolated *Lactobacillus* spp. (isolate-2), that was also able to survive in gastric juice environment at pH 2.2. The bile salt resistance test results of the FSA project by Gibson et al. (year anonymous) provided evidence of the bile tolerance nature of some of the *Lactobacillus* spp. Elizete and Carlos (2005) stated that bile tolerance is an essential characteristic for better survival of LAB, not necessary for multiplication. Schillinger and Lucke (1987) found that the growth of lactobacilli occurred in the presence of 7.5% NaCl isolated from meat and meat products. The NaCl test results of the present study were also similar to Hoque et al. (2010) who isolated *Lactobacillus* spp. from yoghurt samples and tested different concentrations of NaCl (1 to 10%) and found 1 to 9% NaCl tolerance of their *Lactobacillus* spp. Rahman et al. (2015) also found the same result from chicken feces samples LAB isolates in NaCl tolerance assay. The bile salt hydrolase activity test results of the present study were similar to Dashkevicz and Feighner (1989).

According to World Health Organization (WHO) and Food and Agriculture Organization (FAO, 2002), working group guidelines, the probiotic organisms should possess the characteristics of resistance to gastric acid and bile with other attributed criteria. The present experimental outcome revealed that the isolated probiotic lactic acid bacteria have shown similar characteristics/ criteria defined by WHO and FAO standard. Furthermore, the best isolates could have the potential to be used for improved probiotic product development and community based establishment of probiotic product industries to empower the local people and poverty alleviation.

Abbreviation

MRS, De-Man Rogosa Sharpe.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Bartelt M (2000). Diagnostic bacteriology, a study guide. F. A. Davis Co., Philadelphia, PA.

Belicova A, Mikulasova M, Dusinosky R (2013). Probiotic potential and safety properties of *Lactobacillus plantarum* from Slovak Bryndza Cheese. Biomed Res. Int. Article ID 760298, 8 pages.

Berg RD (1998). Probiotics, prebiotics or “conbiotics.” Trends Microbiol. 6:89-92.

Bourlioux P, Pochart P (1988). Nutritional and health properties of yogurt. World Rev. Nutr. Diet 56:21-58.

Chandan RC, Shahani KM (1993). Yogurt. In: Hui YH, edn. Dairy science and technology handbook. New York: VCH Publishers Inc. 1-5.

Collins JK, Thornton G, O’Sullivan GO (1998). Selection of probiotic strains for human applications. Int. Dairy J. 8:487-490.

Dashkevicz M, Feighner SD (1989). Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl. Environ. Microbiol. 55:11-16.

Dave I, Shah NP (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* sp. *bulgaricus, Lactobacillus acidophilus*, and *Bifidobacteria*. J. Dairy Sci. 79:1529-1536.

De Man, JC, Rogosa M, Sharpe ME (1960). A medium for cultivation of *Lactobacilli*, J. Appl. Bacteriol. 23:130-135.

Difco (1998). Difco manual, 11th ed. Difco Laboratories, Detroit, MI.

DNR (Danone Nutricia Research) (2014). History of yoghurt. The nutriJournal: Latest news on health and food. Danone Nutricia Research. Route Départementale 128 - 91767 Palaiseau Cedex, France. http://nutrijournal.danone.com/en/articles/stories/history-of-yogurt/

Driessen FM, Ubbels J, Stadhouders J (1977). Continuous manufacture of yogurt. I. Optimal conditions and kinetics of the prefermentation process. Biotechnol. Bioeng. 19:821-839

Elizete DFRP, Carlos SR (2005). Biochemical characterization and identification of probiotic *Lactobacillus* for swine. B.CEPPA. Curitiba 23:299-310.

Erdourul Z, Erbulur F (2006). Isolation and Characterization of *Lactobacillus bulgaricus* and *Lactobacillus casei* from various foods, Turk. J. Biol. 30:39-44.

Erkus O (2007). Isolation, phenotypic and genotypic characterization of yoghurt starter Bacteria, MS thesis, Izmir Institute of Technology, Turkey.

FAO & WHO (2002). Joint FAO and WHO working group report on Drafting Guidelines for the Evaluation of Probiotics of Food in London, Ontario, Canada. pp. 1-11.

Forbes BA, Sahm DF, Weissfeld AS (2007). Bailey and Scott’s diagnostic microbiology, 12th ed. Mosby Company, St. Louis, MO.

Gibson G R, Rouzaud G, Brostoff J, Rayment N (Year anonymous). An evaluation of probiotic effects in the human gut: microbial aspects. Final Technical report for FSA project. University of Reading and King’s College, London. pp. 1-9.

Goktepe I, Juneja VK, Ahmedna M (2006). Probiotics in food safety and human health. Boca Raton, USA: CRC Press, Taylor & Francis Group.

Graciela FDV, Maria PT (2001). Food Microbiology Protocols (J.F.T. Spencer and A.L.R.D. Spencer, Ed.), Totowa, New Jersey: Humana Press Inc., chap. 21 (Probiotic Properties of *Lactobacillus*). pp. 173-181.

Hartemink MR, Domenech VR, Rombouts FM (1997). LAMVAB – A new selective medium for the isolation of *Lactobacilli* sp. from faces. J. Microbiol. Methods 29:77-84.

Hempel S, Newberry S, Maher AR, Wang Z, Miles JNV, Shanman R, Johnsen B, Shekelle PG (2012). Antibiotic-associate probiotics for the prevention and treatment of antibiotic-associated-diarrhea: a systematic review and meta-analysis. JAMA 307:1959-1969.

Hoque MZ, Akter F, Hossain KM, Rahman MSM, Billah MM, Islam KMD (2010). Isolation, identification and analysis of probiotic properties of *lactobacillus* sp. from selective regional yoghurts. World J. Dairy Food Sci. 5:39-46.

Horne DS (1999). Formation and structure of acidified milk gels. Int. Dairy J. 9:261-268.

International Diary Federation (IDF) (1998). Yoghurt: Enumeration of characteristic microorganisms. Colony count technique at 37°C. International Dairy Federation Bulletin 117A, Brussels, Belgium: 1-5.

Karna BKL, Emata OC, Barraquio VL (2007). Lactic acid and probiotic bacteria from fermented and probiotic dairy products. Science Diliman 19:23.

Linn ZM, Aye KN, Yu SS (2008). In: Processing of Probiotic Yogurt. GMSARN International Conference on Sustainable Development: Issues and Prospects for the Greater Mekong Subregion (GMS), 12-14. Kunming, China.

MacFaddin JF (2000). Biochemical tests for identification of medical bacteria, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, PA.

Martini MC, Lerebours EC, Lin WJ, Harlander SK, Berrada NM, Antoine JM, Savaiano DA (1991). Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on *in vivo* lactose digestion. Am. J. Clin. Nutr. 54:1041-1046.

O’Sullivan MG, Thornton GM, O’Sullivan GC, Collins JK (1992). Probiotic bacteria: myth or reality? Trends Food Sci. Technol. 3:309-314.

Ouwehand A, Salminen S, Isolauri E (2002b). Probiotics: an overview of beneficial effects. potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. Lett. Appl. Microbiol. 26:333-337.

Ouwehand AC, Salminen S, Isolauri E (2002a). Probiotics: An overview of beneficial effects. Antonie van Leeuwenhoek 82:279-289.

Pereira DIA, McCartney AL, Gibson GR (2003). An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. Appl. Environ. Microbiol. 69:4743.

Rahman SMK, Das BK, Soikat FK, Rahman SM, Hossain KM (2015). Characterization of probiotic bacteria isolated from regional chicken feces. Afr. J. Microbiol. Res. 9:880-885.

Reller LB, Mirrett S (1975). Motility-indole-lysine medium for presumptive identification of enteric pathogens of *Enterobacteriaceae*. J. Clin. Microbiol. 2:247-252.

Saarela, M, Lathenmaki L, Crittenden R, Salminen S, Mattilasandholm T (2002). Gut bacteria and health foods - the European perspective. Int. J. Food Microbiol. 78:99-117.

Saccaro DM, Hirota CY, Tamime AY, Oliveira de MN (2011). Evaluation of different selective media for enumeration of probiotic micro-organisms in combination with yogurt starter cultures in fermented milk. Afr. J. Microbiol. Res. 5:3901-3906.

Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I (1998). Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80 Suppl. 1:S147.

Schaeffer AB, Fulton M (1933). A simplified method of staining endospores. Science 77:194.

Schillinger U, Lucke FK (1987). Identification of *Lactobacilli* from meat and meat products. Food Microbiol. 4:199-208.

Sfakianakis P, Tzia C (2014). Conventional and Innovative Processing of Milk for Yogurt Manufacture; Development of Texture and Flavor: A Review. Foods 3:176-193.

Stiles ME, Holzapfel WH (1997). Review article: Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36:1-29.

Xanthopoulos V, Litopoulou-Tzanetaki E, Tzanetakis N (2000). Characterization of *Lactobacillus* isolates from infant faeces as dietary adjuncts. Food Microbiol. 17:205-215.

Zinedine A, Faid M (2007). Isolation and characterization of strains of *Bifidobacteria* with probiotic properties *in vitro*. World J. Dairy Food Sci. 2:28-34.

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Full Length Research Paper

Phenotypic and genotypic methods for detection of metallo beta lactamases among carbapenem resistant Enterobacteriaceae clinical isolates in Alexandria Main University Hospital

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Dissemination of carbapenem resistant Enterobacteriaceae (CRE) poses a considerable threat to public health. The aim of the present work was to estimate the prevalence of metallo beta lactamases (MBL) among CRE isolated from Alexandria Main University Hospital, Egypt, to evaluate the performance of different phenotypic methods for the detection of MBL, and to investigate the local antimicrobial sensitivity profile of these isolates. Eighty CRE were tested for MBL production by Etest® MBL MP/MPI, EDTA double disc synergy test, and EDTA combined disc test. All isolates were confirmed as MBL producers by polymerase chain reaction (PCR). Antibiotic sensitivity testing was performed using disc diffusion method. Among the 80 CRE, 56 isolates (70%) were MBL by PCR. Fifty-four isolates were positive for NDM (96.4%). Meropenem EDTA CDT was the most sensitive test (94.6%). Blood was the most frequent sample from which MBL were isolated (51.7%). Majority of the isolates were isolated from intensive care units (82.1%). All MBL were multidrug resistant; Colistin and polymyxin B showed the lowest resistance rate (26.8 and 19.6%, respectively). The EDTA-CDT will provide a reliable, convenient, and cost-effective approach for detection of MBL in laboratories, which cannot afford to perform molecular tests.

Key words: Metallo beta lactamases, carbapenem resistant Enterobacteriaceae, New Delhi lactamase.

INTRODUCTION

Multidrug resistance is emerging worldwide at an alarming rate among a variety of bacterial species, including the family Enterobacteriaceae (EB) (IDSA et al., 2011). EB members have the tendency to spread easily between humans and to acquire genetic material through horizontal gene transfer, mediated by plasmids and transposons (Nordmann et al., 2011a).

The rapid dissemination of extended spectrum beta lactamases (ESBL) in EB has led to the increased utilization of carbapenems in clinical practice. This is largely because ESBL-producers are capable of hydrolyzing all beta lactams except carbapenems, thus making carbapenems the last option for the treatment of serious infections associated with these organisms

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(Paterson and Bonomo, 2005).

As a consequence of increased use of carbapenems has been the emergence of isolates coding for carbapenemases (Nordmann et al., 2011a). Carbapenem-hydrolysing β-lactamases belong mainly to three clinically significant Amber classes, namely Ambler class A (*Klebsiella pneumoniae* carbapenemases; KPCs), class B (metallo-beta-lactamases; MBLs) and class D (oxacillinases) (Queenan and Bush, 2007).

MBL are broad-spectrum and hydrolyse all beta lactams except monobactams and they are not susceptible to therapeutic β-lactams inhibitors such as clavulanate, sulbactam, and tazobactam. MBLs require zinc-ions to catalyze the hydrolysis of beta-lactam antibiotics and due to the dependence on zinc ions; MBL catalysis is inhibited in the presence of metal-chelating agents like ethylenediaminetetraacetic acid (EDTA) (Bush and Jacoby, 2010). They are mostly found in *K. pneumoniae* isolates and are also frequently associated with serious nosocomial infections and outbreaks (Poirel et al., 2007).

Verona integron-encoded MBLs (VIM) and active on imipenem (IMP) were the common MBLs identified in EB (Nordmann and Poirel, 2002). New Delhi b-lactamase (NDM-1), which originated in India, was first reported in 2009 (Yong et al., 2009), and has been isolated in Europe, Asia, North America, Australasia, and Middle East (Grundmann et al., 2010; Wu et al., 2010; CDC, 2010; Poirel et al., 2010a; Shibl et al., 2013).

Although different phenotypic methods have been described, the Clinical and Laboratory Standards Institute (CLSI) currently does not include standardized recommendations for MBL screening. Carbapenemase gene detection by PCR is considered the gold standard, but its accessibility is often limited to reference laboratories (Behera et al., 2008). Other non-molecular based techniques have been studied; all depend on inhibition of carbapenemase activity by chelating agents e.g. EDTA, dipicolinic acid, and thiol compounds (Galani et al., 2008b).

The aim of the study was to: (1) Estimate the prevalence of MBL-among the tested carbapenemase producing EB isolated from Alexandria Main University Hospital (AMUH), Egypt. 2) Evaluate the performance of different phenotypic methods for the detection of MBL-producing EB in comparison with the gold standard PCR for MBL genes, in order to select a rapid, reliable, economical, and easy to set up workflow method for detection of MBLs. 3) Describe the local antimicrobial sensitivity profile of MBL producing EB isolates aiming to establish an appropriate empirical treatment.

MATERIALS AND METHODS

Clinical isolates

A total of 706 clinical isolates of EB isolated from blood, biological fluids, urine, bronchoalveolar lavage, pus and sputum specimens from patients admitted to various wards and intensive care units in AMUH, were included in a descriptive cross sectional study. The study was conducted for a six-month period starting from January through July 2015.

All isolates were identified by conventional microbiological methods (Tille et al., 2013). All EB isolates were subjected to initial screening for carbapenem resistance byertapenem (ETP) 10 ug, meropenem (MEM) 10 ug, and imipenem (IPM) 10 ug discs, by disc diffusion method and by testing IPM MIC using the broth microdilution method according to CLSI guidelines (CLSI, 2015). Only 80 carbapenem resistant isolates (randomly selected covering the 6 months study duration) were tested for MBL production. Two criteria were used for selection of the 80 isolates: (i) an intermediate or resistant susceptibility to one of the carbapenems (MEM, IPM, ETP) and (ii) an IPM MIC of ≥2 ug/ml (intermediate or resistant). *Escherichia coli* ATCC 25922 was used as a control strain. The control strain was run simultaneously with the test organisms.

The 80 isolates were identified to the species level by the use of mass spectrometry, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Seng et al., 2009) (Bruker Corporation) and were then subjected to the following.

Carbapenemase production confirmatory tests

Modified Hodge test (MHT)

It was performed in accordance with the CLSI guidelines (CLSI, 2015). A 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was prepared in broth. A Mueller Hinton agar plate was inoculated as for the routine disk-diffusion procedure. The plate was allowed to dry for 10 min. MEM disk was placed in the center of the plate. Using a 10 μL loop, three to five colonies of the test organism grown overnight on a blood agar plate were picked and inoculated in a straight line out from the edge of the disk. The streak was at least 20 to 25 mm in length. Following incubation, Mueller Hinton agar was examined for enhanced growth around the test streak at the intersection of the streak and the zone of inhibition.

Result: Enhanced growth = positive for carbapenemase production.

No enhanced growth = negative for carbapenemase production.

The addition of 100 ug/ml of $ZnSO_4$ in Mueller Hinton agar was performed on the same isolates, to improve the limit of detecting MBL production, as previously described (Girlich et al., 2011).

RAPIDEC® CarbaNP test (BioMerieux)

Only ten randomly selected isolates were further tested by RAPIDEC® carbaNP test according to the manufacturer's instructions.

MBL production detection tests

Phenotypic tests

Test isolates' suspensions were adjusted to turbidity equivalent to that of a 0.5 McFarland standard and used to inoculate Mueller-Hinton agar plates.

(a) Etest® MBL MP/MPI was done according to the manufacturer's instructions (BioMérieux, Marcy l'Etoile, France) (Galani et al., 2008b).
(b) EDTA double disc synergy test (EDTA –DDST): Two antibiotic

(MEM 10 ug, IPM 10ug) discs were placed at a distance of 10 mm from a blank filter paper disc (6 mm in diameter, Whatman filter paper no. 2) to which 10 ul of 0.5 M EDTA solution (Sigma-Aldrich, Germany) was added. After overnight incubation, the presence of any synergistic inhibition zone was interpreted as positive (Galani et al., 2008b).

(c) EDTA combined disc test (EDTA-CDT): Two 10 ug MEM discs and Two 10 ug IPM discs were placed on a plate inoculated with the test organism, and 10 ul of 0.5 M EDTA solution was added to one disc of MEM or IPM. The inhibition zones of the MEM and MEM+EDTA or IPM and IPM+EDTA disc were compared after overnight incubation. A zone diameter difference between any of the discs alone and with EDTA ≥7 mm was interpreted as positive (Galani et al., 2008b).

Genotypic tests (Multiplex PCR)

DNA extraction and multiplex PCR amplification for the simultaneous detection of NDM, VIM, and IMP MBL genes was carried out, as previously described, on a thermal cycler instrument (Techne Genius, Cambridge, UK) (Doyle et al., 2012).

Three primer pairs were used to target 3 MBL genes: NDM F: 5’-GCAGCTTGTCGGCCATGCGGGC-3’, NDM R: 5’ GGTCGCGAAGCTGAGCACCGCAT-3’, VIM F:5’-GTTTGGTCGCATATCGCAAC-3’, VIM R: 5’AATGCGCAGCACCAGGATAG-3’, IMP F: 5’-GAAGGCGTTTATGTTCATAC-3’, IMP R: 5’-GTACGTTTCAAGAGTGATGC-3’(Invitrogen by life technologies, Thermo Fisher Scientific Inc. USA).

Multiplex PCR amplification for the simultaneous detection of NDM, VIM, and IMP MBL genes was carried out on a thermal cycler instrument (Techne Genius, Cambridge, UK) using the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 60 s, then final extension of 72°C for 8 min. The PCR reaction consisted of 12.5 µl DreamTaq™ Green PCR Master Mix (2X) (Fermentas), 0.4 uM NDM primer, 0.3 uM each VIM and IMP primer, and PCR grade water to a final volume 25 ul. *E. coli* ATCC 25922 was used as negative control. The PCR products were electrophoresed for 45 min and visualized under UV light.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing of the 80 CRE isolates was performed by the disc diffusion method using Mueller–Hinton agar. The following antibiotics were tested: ampicillin, piperacillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/ tazobactam, cefoperazone/sulbactam, cefaclor, cefuroxime, ceftriaxone, ceftazidime, cefotaxime, cefepime, aztreonam, ciprofloxacin, levofloxacin, ofloxacin, amikacin, gentamicin, tobramycin, chloramphenicol, sulfamethoxazole-trimethoprim, tetracycline, doxycycline, minocycline, tigecycline, tienam, meropenem, ertapenem, colistin and polymyxin B. Norfloxacin, nitrofurantoin, and fosfomycin were only used for urinary isolates. All the antibiotic discs were procured from oxoid, UK. The results were interpreted as per CLSI guidelines (CLSI, 2015), except for colistin and tigecycline. The results for colistin were interpreted by following the criteria proposed by Galani et al. (2008a), and for tigecycline by the breakpoints for EB as suggested by The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). *E. coli* ATCC 25922 was used as a control strain.

ESBL producers were identified by CLSI confirmatory test using a disc of cefotaxime (30 ug) and a disc of cefotaxime/clavulanate (30/10 ug). ESBL production was confirmed if the zone given by the cefotaxime/clavulanate disc was ≥5 mm larger than the zone given by cefotaxime alone (CLSI, 2015).

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 22.0. Analysis of data was done using count percentage. The sensitivity, specificity, positive and negative predictive values and accuracy of the phenotypic MBL detection methods was evaluated using PCR as the gold standard.

Sensitivity = a ×100/a+c,
Specificity = d ×100/b+d,
Positive predictive value = a ×100/a+b,
Negative predictive value = d×100/c+d,
Accuracy = (a+d) ×100/a+b+c+d,

where a= True positives, b=False Positives, c=False Negatives, d=True Negatives (Ilstrup et al., 1990).

RESULTS

During the study period, 706 EB isolates were delivered to the Microbiology Laboratory of AMUH. A total of 240 isolates (33.9%) were initially identified as carbapenem resistant on the basis of their reduced susceptibility to MEM, IPM or ETP by disc diffusion test. Eighty carbapenem resistant EB (CRE) isolates were randomly selected for testing MBL production.

Out of 80 CRE isolates, 75 isolates (93.75%) were *K. pneumoniae*, three isolates (3.75%) were *E. coli* and two isolates (2.5%) were *Enterobacter cloacae* by MALDI-TOF. The highest rate of CRE isolates was isolated from blood cultures (41/80; 51.2%), followed by respiratory cultures (bronchoalveolar lavage, mini BAL, sputum) (25/80; 31.2%), urine (11/80; 13.8%), and pus cultures (3/80; 3.8%). Most of isolates (71/80; 88.75%) were from ICUs followed by internal medicine wards (7/80; 8.75%), then surgical wards (2/80; 2.5%). The mean age of patients was 46 years. Thirty five (44%) were males and 45 (56%) were females (male: female ratio; 1:1.3).

All CRE isolates were at least resistant to one of the carbapenems (IPM, MEM, ETP) by disc diffusion test. Three isolates (3.75%) were sensitive to each of IPM and MEM, while only one isolate (1.25%) was sensitive to ETP. Nine isolates (11.25%) were intermediate to IPM, while only one isolate (1.25%) was intermediate to MEM. Regarding the IPM MIC results, 72 isolates (90%) had an IPM MIC of 8 µg/ml (resistant) and three isolates (3.75%) had an MIC of 4 µg/ml (intermediate), while five isolates (6.25%) were sensitive by MIC (≤1 ug/ml). All sensitive and intermediate isolates were resistant to at least one of the carbapenems by disc diffusion.

The MHT revealed 72 isolates (90%) as positive and eight isolates (10%) as negative for carbapenemase production. Out of the ten isolates tested by RAPIDEC® CarbaNPtest, nine (90%) gave a positive reaction (Table 1 and Figure 1).

Presence or absence of MBL genes was considered the reference method for detection of MBL producing

Table 1. The results of the ten CRE isolates tested by RAPIDEC® CarbaNP test.

Isolate number	MHT	E-test® MBL	MEM EDTA DDST	IPM EDTA DDST	MEM EDTA CDT	IPM EDTA CDT	MBL PCR	RAPIDEC® CarbaNP test
1	+	-	+	+	+	+	+VIM	+
2	+	+	+	+	+	+	+NDM	+
3	+	-	+	+	+	+	+NDM	+
4	+	+	-	-	-	-	+NDM	+
5	-	+	+	+	+	+	+NDM	-
6	+	-	-	-	-	-	-	+
7	+	+	-	-	-	-	-	+
8	+	-	-	+	-	-	-	+
9	+	-	-	+	-	-	-	+
10	+	-	-	-	+	-	-	+

+: Positive, -: negative.

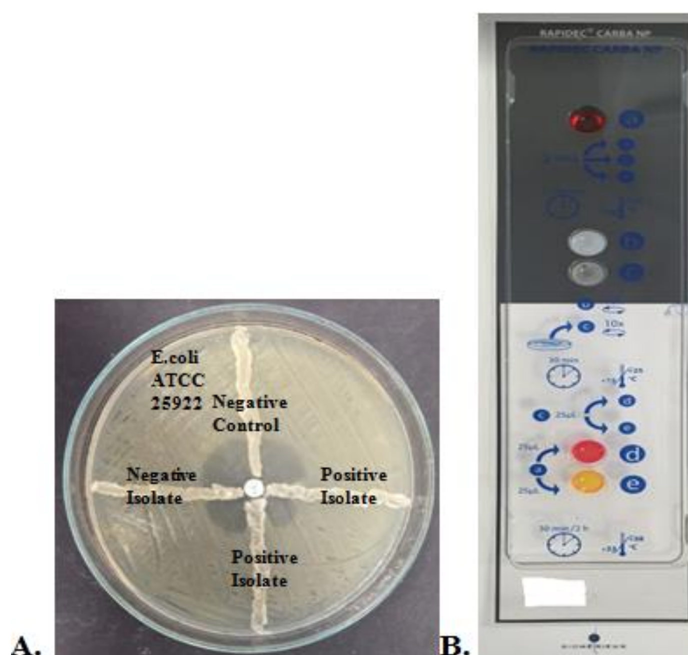

Figure 1. A. Results of MHT. B. Results of RAPIDEC® CarbaNP test (BioMerieux): a positive carbapenemase test.

CRE. Accordingly, among the selected 80 CRE, 56 isolates (56/80; 70%) were identified as MBL producing CRE. The distribution of MBL genes was as followed: 54 isolates were positive for NDM (67.5% from all CRE and 96.4% from MBL producing CRE), two positive for VIM (2.5% of all CRE and 3.6% of MBL producing CRE), and none of the isolates harbored IMP gene. Fifty-four MBL isolates (96.4%) were *K. pneumoniae* and two (3.6%) were *Enterobacter cloacae* (one harboring VIM gene and the other the NDM gene (Figure 2).

Out of the 56 PCR positive isolates, 50 (89.3%) were MHT positive and six (10.7%) were negative. Eight of the positive isolates (14.3%) gave a weakly positive reaction. The six negative MHT isolates were retested after addition of zinc and three isolates showed a positive test increasing the sensitivity of the test from 89.3 to 94.6%.

Regarding the results of phenotypic tests, the E-test® MBL MP/MPI identified 46 isolates (57.5%) as MBL producers, while the EDTA-DDST identified 60 isolates (75%) by IPM EDTA-DDST, and 54 isolates (67.5%) by MEM EDTA-DDST. The EDTA-CDT identified 67 isolates (83.75%) by MEM EDTA-CDT, and 55 isolates (68.75%) by IPM EDTA-CDT (Figure 3 and Table 2) summarizes the results of all phenotypic tests in comparison with MBL

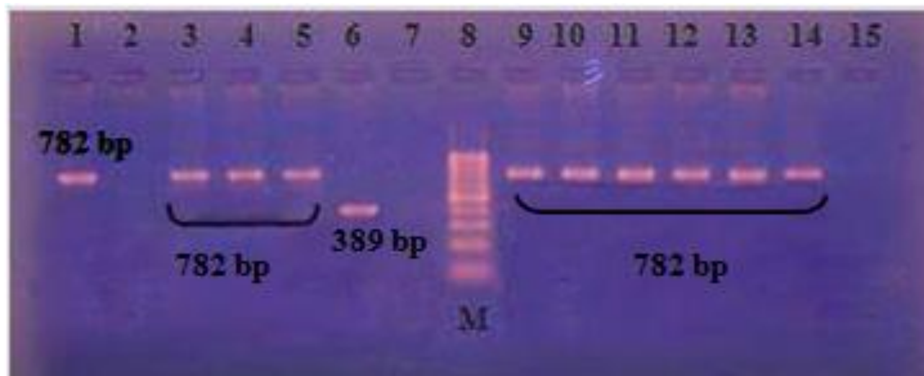

Figure 2. Agarose gel stained with ethidium bromide of MBL isolates. Lane 1, 3-5, 9-14: show positive amplified NDM gene (782 bp). Lane 6: shows VIM gene (389 bp). Lane 2 and 7: show negative results. Lane 15: negative control. Lane 8: 100-1000 bp DNA ladder.

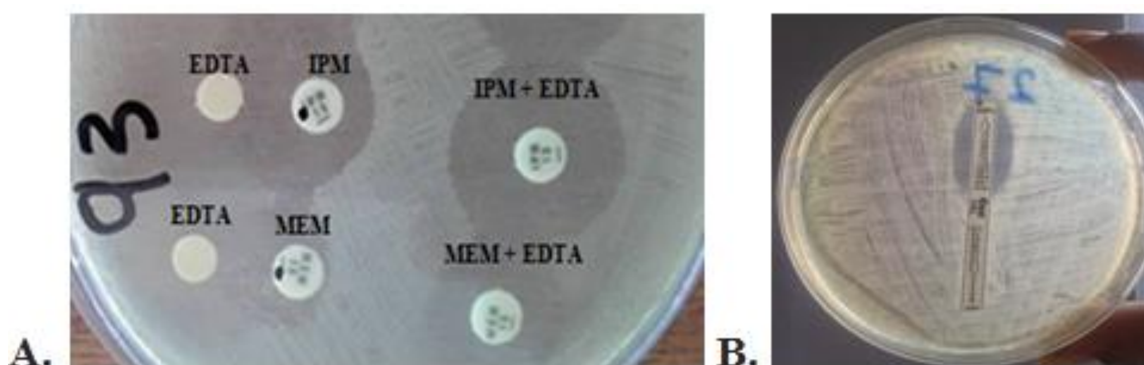

Figure 3. (A) Results of double disc and combined discs for testing of MBL. (B) Etest® MBL MP/MPI showing positive result.

Table 2. Results of all phenotypic tests in comparison to PCR for the 80 CRE isolates.

Phenotypic tests	MBL +ve (n=56)		MBL –ve (n=24)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
	True +ve	False -ve	True -ve	False +ve					
E-test® MBL	45	11	23	1	80.3	95.8	97.8	67.6	85
MEM EDTA DDST	45	11	15	9	80.3	62.5	83.3	57.7	75
IPM EDTA DDST	45	11	9	15	80.3	37.5	75	45	67.5
MEM EDTA CDT	53	3	10	14	94.6	41.6	79.1	76.9	78.75
IPM EDTA CDT	51	5	20	4	91.1	83.3	92.7	80	88.75

MBL +ve: MBL positive, MBL –ve: MBL negative. PPV: positive predictive value, NPV: negative predictive value.

gene detection by PCR. None of the tests was 100% sensitive orspecific.

The results of screening tests for carbapenemases (carbapenem disc diffusion, IPM MIC, MHT, carbaNP test) could not be included in the comparison to gold standard PCR results as they are used for screening of all types of carbapenemases and they are not specific for MBL only.

Concerning the antibiotic susceptibility profile, all MBL isolates (100%) were ESBL producers as detected by the CLSI confirmatory test, and all isolates (100%) were multidrug resistant (resistant to three or more antibiotic classes). All isolates (100%) were resistant to penicillins and cephalosporins except one isolate (98.2%) which

Table 3. The distribution of antimicrobial resistance of the 56 MBL isolates.

Antibiotic	Resistance	
	No	%
Meropenem	54	96.4
Ertapenem	55	98.2
ipenemIm	51	91
Ampicillin	56	100
Piperacillin	56	100
Cefaclor	56	100
Cefuroxime	56	100
Ceftriaxone	56	100
Ceftazidime	56	100
Cefotaxime	56	100
Cefepime	55	98.2
Aztreonam	55	98.2
Amoxicillin/clavulanic acid	56	100
Ampicillin/sulbactam	56	100
Piperacillin/tazobactam	54	96.4
Cefoperazone/sulbactam	53	94.6
Gentamicin	49	87.5
Amikacin	50	89.3
Tobramycin	51	91
Tetracycline	56	100
Doxycycline	44	78.6
Minocycline	44	78.6
Tigecycline	19	33.9
Ciprofloxacin	44	78.6
Levofloxacin	25	44.6
Ofloxacin	56	100
Trimethoprim-sulphamethoxazole	51	91
Chloramphenicol	40	71.4
Colistin	15	26.8
PolymyxinB	11	19.6
For urine isolates only (n=7)		
Norfloxacin	7	100
Nitrofurantoin	3	42.8
Fosfomycin	0	0
Combined Resistance		
Cephalosporins + fluoroquinolones + carbapenems	6	10.7
Cephalosporins + aminoglycosides + carbapenems	28	50
Cephalosporins + fluoroquinolones + aminoglycosides + carbapenems	19	33.9

was sensitive to each of cefepime and aztreonam. Antibiotic resistance pattern is as shown in Table 3.

Regarding the distribution of MBL positive isolates among the different clinical specimens, blood was the most frequent sample from which MBL were isolated (29 isolates, 51.7%), followed by respiratory samples (16 isolates, 28.6%), urine (8 isolates, 14.3%) and pus (3 isolates, 5.4%). Majority of MBL isolates were isolated from ICUs (46 isolates) representing 82.1%. Five isolates (8.9%) were from hematology ward. One isolate (1.8%) was isolated from each of the following wards (rheumatology, diabetes and metabolism, hepatology, plastic and burn unit). The mean age of patients was 46 years with male: female ratio 1:1.3.

DISCUSSION

According to data from the European Antimicrobial Resistance Surveillance Network (EARS Net, formerly EARSS, 2014), the population-weighted mean for carbapenem resistance was 8.3% in 2013 (ECDC, 2014). The percentages of resistant isolates in the reporting countries ranged from 0% (Bulgaria, Finland, Iceland, Latvia, Lithuania and Sweden) to 59.4 % (Greece).

Out of 594 *K. pneumoniae* isolates, 5.6% were carbapenem resistant, according to US Naval Medical Research Unit No 3, Global Disease Detection Program, conducted in Egypt as part of the National surveillance 2002 to 2010 (WHO, 2014). A higher prevalence was in the present study (33.9%). This could be explained by the continuous use of carbapenems in treatment due to high prevalence of ESBL strains in our hospital.

In the present study, a very high rate of MBL (56/80; 70%) was reported among EB, of these 96.4% was *K. pneumoniae* and 3.6% was *E. cloacae*. Several studies also demonstrated increasing incidence of MBL production in EB isolates (Yong et al., 2006; Datta et al., 2012).

In the present study, the MHT had a sensitivity of 94.6% for detecting MBL producers. This sensitivity is higher than that reported by Doyle et al. (2012) (only 12%). The MHT and MHT added with $ZnSO_4$ showed a positive result, respectively, for 72 and 75 of the 80 CRE isolates. Similarly, Ambretti et al. (2013) showed better MHT results after addition of zinc. This finding should be taken into consideration while detecting MBL in routine work.

For financial reasons, CarbaNP was performed for only ten CRE isolates, the sensitivity of the test (90%) was less than reported elsewhere (Nordmann et al., 2012). The negative carbaNP isolate (NDM MBL PCR positive) was also negative for MHT, this could explain negative results, however, the results obtained from this isolate need further assessment. The positive results obtained with MBL PCR negative isolates could be explained by the presence of other types of carbapenemases as positive results were obtained in less than 30 min with the five negative isolates.

According to the results of multiplex PCR, 56 out of 80 (70%) CRE were MBL producers, NDM gene was detected in 96.4% of the isolates. Similarly, in a study conducted in Pakistan, gene for NDM-1 enzymes was detected in 94% of clinical isolates and none of the clinical isolates were found positive for IMP, VIM and KPC enzymes (Sultan et al., 2013). On the other hand, *K. pneumoniae* isolates with VIM-MBLs have been found as causes of country wide epidemics in the USA, several Latin American countries, China and Europe (Grundmann et al., 2010).

NDM-1 is the latest carbapenemase to be discovered. It was first described in 2008 in *K. pneumoniae* and *E. coli* isolated in Sweden from an Indian patient transferred from a New Delhi hospital (Yong et al., 2009). Most positive NDM bacterial isolates have shown epidemiological links to India and Pakistan (Nordmann et al., 2011b). It has been suggested that the Middle East region might be a secondary reservoir for the spread of NDM-1 isolates as there is a high frequency of population movement between the region and the Indian subcontinent (Nordmann et al., 2011b). NDM CRE have been reported in Oman (Poirel et al., 2010b), Kuwait (Jamal et al., 2011), Saudi Arabia (Shibl et al., 2013) and Morocco (Poirel et al., 2011). Of interest, the majority of the cases could not be directly linked to the Indian subcontinent nor had a history of foreign travel.

The first reported case of NDM MBL CRE in Egypt was in a cancer patient in Cairo, in 2012, by Abdelaziz et al. (2013). There was no apparent epidemiological link to an endemic area. The study reinforced the hypothesis of an autochthonous presence of the NDM resistance determinant in the Middle East and North African area. To the best of our knowledge, this is the first report of NDM carrying *K. pneumoniae* in Alexandria, Egypt.

In the present study, blood was the most frequent sample from which MBL were isolated (51.7%). Majority of MBL isolates were isolated from ICUs (82.1%). Our findings are in accordance with several studies, which found that the majority of clinical isolates were yielded from blood cultures and from patients in the ICU (Bora et al., 2014; Sultan et al., 2013; Shibl et al., 2013).

Out of three phenotypic methods used for confirmation of MBL production, the overall sensitivity of EDTA-CDT was better than that of E-test and EDTA-DDST. Previous studies also found that the combined disc test was a highly sensitive (100%) method among the analyzed procedures (Picao et al., 2008; Galani et al., 2008b; Bora et al., 2014). This could be attributed to the subjective interpretation of DDST results. It should be mentioned that a 7 mm was used as a cutoff in CDT (Galani et al., 2008b) and that our results of CDT would be definitely better (much sensitive test) if relied on a smaller diameter difference between EDTA free and EDTA combined discs.

The MBL IPM E-test was designed to detect the presence of MBLs in *P. aeruginosa* (Walsh et al., 2002). This E-test is often difficult to interpret when investigating the presence of MBLs in EB. This is due to the fact that the MICs of IPM are often low in EB that produce MBLs (Doyle et al., 2012). For this reason and as recommended by BioMérieux, the E-test was performed using MEM and not IPM EDTA strips. These results of this study were much better in term of sensitivity (80.3%) and specificity (95.8%) than that reported previously by authors using IPM EDTA strips (Doyle et al., 2012; Galani et al., 2008b).

MBL inhibitor (EDTA) may possess their own bactericidal activity, which may result in expanded inhibition zones not associated with true MBL production and hence false positive results (Chu et al., 2005). On the

other hand, authors reported that false-negative results might arise from carbapenem hydrolysis or inactivation caused by EDTA (Picao et al., 2008). Also, previous studies reported that phenotypic tests failed to identify the presence of MBL in isolates harboring more than one carbapenemase gene (Bartolini et al., 2014). This could justify false positive and negative results of all phenotypic tests encountered in our study.

NDM gene is carried on plasmids which also carry a number of other genes conferring resistance to aminoglycosides, macrolides and sulphamethoxazole, thus making these isolates multidrug resistant (Franklin et al., 2006). This is in agreement with the findings of the present study, which revealed that all isolates were multidrug resistant. Combined resistance to cephalosporins, aminoglycosides and carbapenems was the most frequent resistance phenotype (50%) encountered in the study. The antibiotic susceptibility profile of our isolates is in keeping with the reported multidrug-resistant phenotype associated with isolates harboring NDM (ECDC, 2014; Shibl et al., 2013; Poirel et al., 2011).

Treatment of patients infected with MBL producers is challenging due to the currently limited options. The tested isolates showed the lowest resistance to fosfomycin, polymyxin B, colistin and tigecycline (0, 19.6, 26.8, and 33.9%, respectively), but these antibiotics also have limitations and adverse effects (El-Herte et al., 2012). Although Colistin and polymyxin B seem to be the last treatment choice for these isolates; our finding of colistin and polymyxin B resistant MBL-producers is of major concern.

On the basis of our study findings, it was concluded that EDTA-CDT could be a sensitive, easy to perform, and interpret phenotypic rapid method for the detection of MBLs in Enterobacteriaceae. It could be introduced into the workflow of any clinical Microbiology laboratory that routinely performs antibiotic sensitivity by disc diffusion test. The liability of subjective interpretation of EDTA-DDST makes it a bad choice. Although the E-test has a higher specificity, it is not considered a cost-effective test.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdelaziz MO, Bonura C, Aleo A, Fasciana T, Mammina C (2012). NDM-1- and OXA-163-producing *Klebsiella pneumoniae* isolates in Cairo, Egypt, 2012. J. Glob. Antimicrob. Resist. 1(4):213-215.

Ambretti S, Gaibani P, Berlingeri A, Cordovana M, Tamburini MV, Bua G, Landini MP, Sambri V (2013). Evaluation of phenotypic and genotypic approaches for the detection of class A and class B carbapenemases in *Enterobacteriaceae*. Microb. Drug Resist. 19(3):212-215.

Bartolini A, Frasson I, Cavallaro A, Richter SN, Palù G (2014). Comparison of phenotypic methods for the detection of carbapenem non-susceptible *Enterobacteriaceae*. Gut Pathog. 6:13.

Behera B, Mathur P, Das A, Kapil A, Sharma, V (2008). An evaluation of four different phenotypic techniques for detection of metallo-β-lactamase producing *Pseudomonas aeruginosa*. Ind. J. Med. Microbiol. 26(3):233-237.

Bora A, Sanjana R, Jha BK, Mahaseth SN, Pokharel K (2014). Incidence of metallo-beta-lactamase producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in central Nepal. BMC Res. Notes 7:557.

Bush K, Jacoby GA (2010). Updated functional classification of b-lactamases. Antimicrob. Agents Chemother. 54:969-979.

Centers for Disease Control and Prevention (CDC) (2010). Detection of *Enterobacteriaceae* isolates carrying metallo-beta-lactamase—United States. MMWR Morb. Mortal. Wkly. Rep. 59:750.

Chu Y W, Cheung TK, Ngan JY, and Kam KM (2005).EDTA susceptibility leading to false detection of metallo-beta-lactamase in *Pseudomonas aeruginosa* by Etest and an imipenem-EDTA disk method. Int. J. Antimicrob. Agents 26:340-341.

Clinical and Laboratory Standards Institute (CLSI) (2015). Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. CLSI document M100-S25 Clinical and laboratory standards institute, Wayne, Pennsylvania, USA. 35:3.

Datta S, Wattal C, Goel N, Oberoi JK, Raveendran R, Prasad KJ (2012). A ten year analysis of multi-drug resistant blood stream infections caused by *Escherichia coli* & *Klebsiella pneumoniae* in a tertiary care hospital. Indian J. Med. Res. 135:907-912.

Doyle D, Peirano G, Lascols C, Lloyd T, Church DL, Pitout JD (2012). Laboratory detection of *Enterobacteriaceae* that produce carbapenemases. J. Clin. Microbiol. 50(12):3877-3880.

ECDC-European Centre for Disease Prevention and Control (2014).Antimicrobialresistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC.

El-Herte RI, Araj GF, Matar GM, Baroud M, Kanafani ZA, Kanj SS (2012). Detection of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* producing NDM-1 in Lebanon. J. Infect. Dev. Ctries. 6:457-461.

Franklin C, Liolios L, Peleg Ay (2006). Phenotypic detection of carbapenem susceptible metallo-β-lactamase producing Gram negative bacilli in the clinical laboratory. J. Clin. Microbiol. 44:3139-3144.

Galani I, Kontopidou F, Souli M, Rekatsina PD, Koratzanis E, Deliolanis J, Giamarellou H (2008a). Colistin susceptibility testing by Etest and disc diffusion methods. Int. J. Antimicrob. Agents 31:434-439.

Galani I, Rekatsina PD, Hatzaki D, Plachouras D, Souli M, Giamarellou H (2008b). Evaluation of different laboratory tests for the detection of metallo-beta-lactamase production in *Enterobacteriaceae*. J. Antimicrob. Chemother. 61(3):548-553.

Girlich D, Poirel L, Nordmann P (2011). Value of the modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*. J. Clin. Microbiol. 50:477-479.

Grundmann H, Livermore DM, Giske CG, Canton R, Rossolini GM, Campos J, Vatopoulos A, Gniadkowski M, Toth A, Pfeifer Y, Jarlier V, Carmeli Y, CNSE Working Group (2010). Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro. Surveill. 15(46). pii: 19711.

Ilstrup DM (1990). Statistical methods in microbiology. Clin. Microbiol. Rev. 3:219-226.

IDSA-Infectious Diseases Society of America, Spellberg B, Blaser M, Guidos RJ, Boucher HW, Bradley JS, Eisenstein BI, Gerding D, Lynfield R, Reller LB, Rex J, Schwartz D, Septimus E, Tenover FC, Gilbert DN (2011). Combating antimicrobial resistance: policy recommendations to save lives. Clin. Infect. Dis. 52(Suppl 5):S397-428.

Jamal W, Rotimi VO, Albert MJ, Khodakhast F, Udo EE, Poirel L (2011). Emergence of nosocomial New Delhi metallo-beta-lactamase-1 (NDM-1)-producing *Klebsiella pneumoniae* in patients admitted to a tertiary care hospital in Kuwait. Int. J. Antimicrob. Agents 39:183-184.

Nordmann P, Naas T, Poirel L (2011). Global spread of carbapenemase producing *Enterobacteriaceae*. Emerg. Infect. Dis. 17:1791-1798.

Nordmann P, Poirel L (2002). Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. 8:321-331.

Nordmann P, Poirel L, Dortet L (2012). Rapid Detection of Carbapenemase producing *Enterobacteriaceae*. Emerg. Infect. Dis. 18:1503-1507.

Nordmann P, Poirel L, Walsh TR, Livermore DM (2011b).The emerging NDM carbapenemases. Trends Microbiol.19(12):588-595.

Paterson DL, Bonomo RA (2005). Extended-spectrum beta-lactamases: a clinical up-date. Clin. Microbiol. Rev. 18:657-686.

Picao RC, Andrade SS, Nicoletti AG, Campana EH, Moraes GC, Mendes RE, Gales AC (2008).Metallo-ß-Lactamase Detection: Comparative Evaluation of Double Disk Synergy versus Combined Disk Tests for IMP-, GIM-, SIM-, SPM-, or VIM- Producing Isolates. J. Clin. Microbiol. 46(6):2028-2037.

Poirel L, Al Maskari Z, Al Rashdi F, Bernabeu S, Nordmann P (2010b). NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. J. Antimicrob. Chemother. 66:304-306.

Poirel L, Benouda A, Hays C, Nordmann P (2011). Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco. J. Antimicrob. Chemother. 66(12):2781-2783.

Poirel L, Lagrutta E, Taylor P, Pham J, Nordmann P (2010a). Emergence of metallo-beta- lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. Antimicrob. Agents Chemother. 54:4914-4916.

Poirel L, Pitout JD, Nordmann P (2007). Carbapenemases: Molecular diversity and clinical consequences. Future Microbiol. 2:501-512.

Queenan AM, Bush K (2007). Carbapenemases: the versatile b-lactamases. Clin. Microbiol. Rev. 20:440-458.

Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Infect. Dis. 49(4):552-553.

Shibl A, Al-Agamy M, Memish Z, Senok A, Khader SA, Assiri A (2013). The emergence of OXA-48- and NDM-1-positive *Klebsiella pneumoniae* in Riyadh, Saudi Arabia. Int. J. Infect. Dis. 17(12):e1130-3.

Sultan BA, Irfan S, Khan E (2013). Detection of Metallo-beta Lactamases (imp, Vim, Ndm) and Kpc carbapenemases in carbapenem resistant *Enterobacteriaceae* and Vntr analysis of *Klebsiella pneumonia* isolates: Report from a Tertiary Care Hospital Karachi, Pakistan. Infect. Dis. J. Pak. 22(3):584-587.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0.

Tille PM. Bailey & Scott's Diagnostic Microbiology (2013).13th edition. Philadelphia, PA, USA: Mosby Elsevier.

Walsh TR, Bolmstrom A, Qwarnstrom A, Gales A (2002).Evaluation of a new Etest for detecting metallo-beta-lactamases in routine clinical testing. J. Clin. Microbiol. 40:275-279.

World Health Organization (2014). Antimicrobial resistance: global report on surveillance. WHO.

Wu HS, Chen TL, Chen IC, Huang MS, Wang FD, Fung CP, Lee SD (2010). First identification of a patient colonized with *Klebsiella pneumoniae* carrying blaNDM-1 in Taiwan. J. Chin. Med. Assoc. 73:596-598.

Yong D, Choi YS, Roh KH, Kim CK, Park YH, Yum JH, Lee K, Chong Y (2006). Increasing prevalence and diversity of metallo-beta-lactamases in *Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacteriaceae* from Korea. J. Antimicrob. Agents Chemother. 50:1884-1886.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (2009). Characterization of a new metallo-beta-lactamase gene, bla (NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother. 53:5046-5054.

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Full Length Research Paper

Molecular detection of resistance to rifampicin and isoniazid in tuberculosis patients in Senegal

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The aim of this study was to use molecular methods to determine the profile of resistance to rifampicin (RMP or RIF) and isoniazid (INH) in mycobacteria from tuberculosis patients in Senegal. Sputum samples (48) received by the mycobacterial laboratory of the National Antituberculosis Program (NATP) in Senegal between 2012 and 2014 were studied. Most of these samples came from patients in treatment failure or relapse (58.33%). They were tested with the Xpert MTB/RIF or line-probe assays (LPAs) or both. 17 (35.41%) isolates resistant to INH, 16 (33.33%) resistant to RMP, and 16 that were multidrug-resistant (MDR) (33.33%) were identified. Two isolates (4.16%) were susceptible to INH, but resistant to RMP (INH-S/RIF-R). The molecular tests facilitated the rapid detection of MDR isolates. However, INH resistance should be assessed in all cases in which RIF resistance is detected, given the demonstrated existence of INH-S/RIF-R strains.

Key words: Tuberculosis, Xpert MTB/RIF, line-probe assays (LPA), resistance, Senegal.

INTRODUCTION

With an annual incidence exceeding 300 cases per 100,000 inhabitants in some countries, particularly in sub-Saharan Africa, tuberculosis remains a major public health problem (Mbatchou et al., 2008). This disease is treated with a combination of four antituberculosis drugs: rifampicin (RMP), isoniazid (INH), ethambutol (EMB), and streptomycin (SM). Multi-resistance to antituberculous drugs (multidrug resistance or MDR) is defined as resistance to both RMP and INH (Kurbatova et al., 2012). The objective of this study was to use molecular methods to determine the profile of resistance to RMP and INH in the mycobacteria present in tuberculosis patients in Senegal.

MATERIALS AND METHODS

Sputum samples received by the mycobacterial laboratory of the

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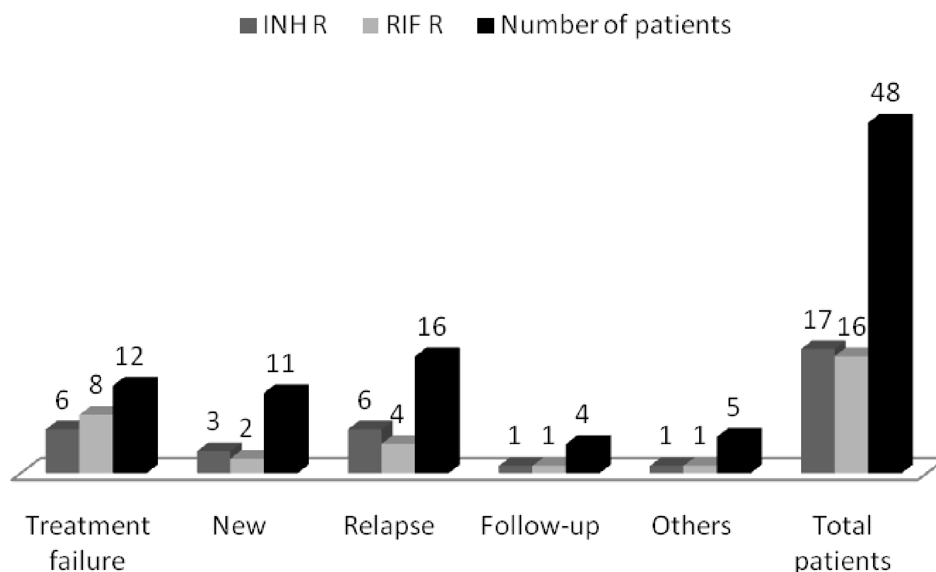

Figure 1. Distribution of strains resistant to RMP and INH by patients category.

Table 1. Classification of the isolates according to Xpert MTB/RIF and LPA results.

LPA	Xpert MTB/RIF		Total
	RIF R	RIF S	
INH-R/RIF-R	13	0	13
INH-S/RIF-R	2	0	2
INH-S/ RIF-S	0	4	4
Total	15	4	19

National Antituberculosis Program (NATP), the national reference laboratory in Senegal, between 2012 and 2014 were studied. Clinical data (full name, age, sex, clinical diagnosis, history of antituberculous treatment, and referring structure) were obtained from the analysis reports accompanying the samples.

The procedure for analyzing these samples in the laboratory began with Ziehl-Neelsen staining and microscopy, to check that the sample contained acid-fast bacteria (AFB). All the samples included in this study yielded positive microscopy results. Two molecular tests recommended by the WHO (WHO Policy Statement (WHO, 2015; Rapid Implementation of Xpert MTB/RIF (WHO), 2011) and able to identify mycobacteria of the tuberculosis complex and the most frequent mutations conferring resistance to RMP and INH were then used: the line-probe assay (LPA) (FIND, 2015) and Xpert MTB/RIF (Rapid implementation of Xpert MTB/RIF (WHO), 2011).

The Xpert MTB/RIF test involves using polymerase chain reaction (PCR) to amplify directly the *rpoB* gene of tuberculosis complex mycobacteria present in the sample. It can also be used to detect the mutations associated with RMP resistance. This test is supplied in the form of ready-to-use cartridges. The sample (2 ml) is decontaminated, neutralized, and diluted in a specific solution and then added to the cartridge. The cartridge is then loaded into the machine, which carries out all the steps of the PCR. The result is obtained after about 2 h.

LPA is a molecular technique that can be used directly on samples to detect mutations of the *katG* and *inhA* genes (conferring high- or low-level resistance to INH) and *rpoB* gene (resistance to RMP). The result is obtained after about 5 h.

Depending on the availability of the reagents, the samples were tested with the Xpert MTB/RIF alone, the LPA alone, or with both. In cases in which both tests were used, the LPA was used principally to check the susceptibility to INH of the isolates resistant to RMP identified by Xpert MTB/RIF. The data were analyzed with Epi-Info version 7.

RESULTS

This study included a total of 48 sputum samples during the period studied. These samples came from patients aged between 9 and 89 years old (mean 36.06); the sex ratio (M/F) was 2.35. Most of these samples came from patients with treatment failure (25%) or relapses (33.33%) (Figure 1). Thirteen (13) of these samples were tested only with the Xpert MTB/RIF, 16 were tested only with the LPA, and 19 were tested with both (Table 1). Of the samples tested with the Xpert MTB/RIF, one was found to be resistant to RMP, 10 were susceptible to RMP and the other two negative. The LPA identified 3 isolates as INH-R/RIF-R, 3 as INH-R/RIF-S, 9 as INH-S/RIF-S and 1 sample to be negative. By combining Xpert MTB/RIF and LPA results, two isolates (4.16%) susceptible to INH and resistant to RMP (INH-S/RIF-R) were identified (Table 1).

In total, 16 MDR isolates were detected (33.33%). The rates of resistance were high among patients with relapses (37.5% resistant to INH and 25% resistant to

RMP) or treatment failure (50% resistant to INH and 66.66% resistant to RMP) (Figure 1).

DISCUSSION

The emergence of resistance to antituberculous drugs is a major problem in most African countries (Sangaré et al., 2010). Two molecular techniques recommended by the WHO and suitable for use directly on samples and in laboratories in the field were used, because they do not require the extensive biosafety precautions needed for culture of the tuberculosis bacillus. In Senegal, the national resistance screening algorithm in use since 2014 involves the use of the Xpert MTB/RIF as the first-line diagnostic tool. Consequently, the NATP has equipped five regions (Kaolack, Saint Louis, Kolda, Fatick, and Diourbel) with Xpert MTB/RIF machines, to facilitate the detection of MDR strains (PNT, 2013). Most of our samples came from young male patients, as reported in other studies (Diop et al., 2014). Most presented treatment failure or relapse (58.33%), potentially accounted for the high frequency of MDR strain detection in this study (33.33%). Indeed, this frequency is higher than that reported for Senegal in 2010 (1% strains in new patients were MDR and 11% in treated patients) (Chevalier et al., 2010). However, it is lower than frequencies reported for the Central African Republic (40%) (Minime-Lingoupou et al., 2010), Burkina Faso (50.5%) (Sangaré et al., 2010) and Ivory Coast (79%) (Kouassi et al., 2004).

Some samples were positive for Ziehl-Neelsen staining, but tested negative with Xpert MTB/RIF or the LPA. This can be explained by the presence of a typical mycobacteria undetectable with these two techniques. Three INH-R/RIF-S isolates were identified, consistent with the suggestion that RMP is often the last antituberculous drug to be affected by resistance; note that the definition of multidrug resistance is the association of resistance to both RMP and INH (Kurbatova et al., 2012). Nevertheless, two isolates (4.16%) resistant to RMP were found, but susceptible to INH (RIF-R/INH-S). Other studies have reported the occurrence of such isolates, including that of Kurbatova et al. (2012), in which data from 14 supranational reference laboratories for cultures carried out in 112 laboratories in 80 countries were analyzed retrospectively. Isolates resistant to RMP and susceptible to INH were identified, with significantly lower levels of resistance to other first- and second-line antituberculous drugs (with the exception of rifabutin) than in MDR strains. A comparison of our results with published findings (Kurbatova et al., 2012) suggested that testing for resistance to RMP may not necessarily be the best approach to the diagnosis of probable MDR tuberculosis, with implications for the use of tests identifying only DNA mutations associated with RMP resistance (Kurbatova et al., 2012). Indeed, the two antibiotics considered here do not act on *Mycobacterium tuberculosis* in the same way. INH has powerful bactericidal activity against *M. tuberculosis*. This prodrug is activated by the KatG enzyme of *M. tuberculosis*, a catalase-peroxidase (Brossier, 2011). INH inhibits the synthesis of the mycobacterial cell wall, leading to cell death. About 80% of the strains resistant to INH carry point mutations or partial or complete deletions of the *katG* gene. Resistance to RMP is conferred by mutations of the *rpoB* gene (Brossier, 2011; Prasad et al., 2014).

Conclusion

With Xpert MTB/RIF and the LPA, high levels of resistance to RMP and INH in the patients studied were found, most of whom suffered from treatment failure or relapse. Xpert MTB/RIF is a high-performance test that is simple to use and can facilitate the detection of strains resistant to RMP, particularly in developing countries. The findings of this study demonstrate that some strains may be resistant to RMP, but susceptible to INH (4.16% in our study), resulting in their misclassification as MDR strains if testing for MDR strains is based exclusively on resistance to RMP. It would therefore be useful, if possible, to test strains identified as resistant to RMP for resistance to INH.

Conflicts of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Brossier F (2011). Mechanisms of action and resistance to INH, a first-line antituberculous drug. J. Anti. infect. 10 (003). http://www.em-consulte.com/article/678308/mecanismes-daction-et-de-resistance-de-lisoniazide.

Chevalier B, Margery J, Sane M, Camara P, Lefebvre N, Gueye M, Thiam M, Perrier-Gros-Claude JD, Simon F, Fabre M, Soler C, Herve V, Mbaye PS, Debonne JM (2010). Epidemiology of the resistance of *Mycobacterium tuberculosis* to antituberculosis drugs at the main hospital in Dakar, Senegal. A 4-year retrospective study (2000-2003). Rev. Pneumol. Clin. 66(4):266-271.

Diop SA, Fortes Déguénonvo L, Manga NM, Dia NM, Ka D, Hounsou AS, Ndour CT, Soumaré M, Seydi M (2014). Epidemiological, clinical and evolutionary aspects of tuberculosis retreatment patients. RMIM. 3:9-13. http://infectiologiemali.org/attachments/article/116/REMIM%20Tome%20III%20p9-13.pdf

FIND (2015). Line probe assay (1st line drugs). http://www.finddiagnostics.org/programs/tb/find_activities/line_probe_assay_1.html.

Kouassi B, Horo K, N'douba KA, Koffi N, Ngom A, Aka-Danguy E, Dosso M (2004). Epidemiological, clinical and biological profile of pulmonary tuberculosis in situation of failure or relapse in Abidjan. Bull.Soc. Pathol. Exot. 97(5):336-337.

Kurbatova EV, Cavanaugh JS, Shah NS, Wright A, Kim H, Metchock B, Van Deun A, Barrera L, Boulahbal F, Richter E, Martin-Casabona N (2012). Rifampicin-resistant Mycobacterium tuberculosis: susceptibility to isoniazid and other anti-tuberculosis drugs. Int. J. Tuberc. Lung Dis. 16 (3):355-357.

Mbatchou NBH, Diatta A, Touré NO, Dia Kane Y, Ba Diop S, Ndiaye EH, Niang A, Sah Belinga A, Agodokpessi G, Ngakoutou R, Thiam K, Hane AA (2008). Clinical, biological and radiological spectrum of newly diagnosed pulmonary tuberculosis. Rev. Mal. Respir. 25:22-26.

Minime-Lingoupou F, Pierre-Audigier C, Kassa-Kélémbho E, Barilone N, Zandanga G, Rauzier J, Cadet-Daniel V, Le Faou A, Gicquel B (2010). Rapid identification of multidrug-resistant tuberculosis isolates in treatment failure or relapse patients in Bangui, Central African Republic. Int.J.Tuberc. Lung. Dis.14(6):782-785.

Prasad R, Gupta N, Singh M (2014). Multidrug-resistant tuberculosis: trends and control. Indian. J. Chest. Dis. Allied. Sci. 56:237-246.

National Antituberculosis Program (PNT), Senegal. Annual Report, 2013. http://www.sante.gouv.sn/index.php?option=com_content&view=article&id=1323&Itemid=784.

Rapid implementation of Xpert MTB/RIF (2011). WHO Report, Geneva. WHO/HTM/TB/ 2011.2. http://apps.who.int/iris/bitstream/10665/44593/1/9789241501569_eng.pdf

Sangaré L, Diandé S, Badoum G, Dingtoumda B, Traoré AS (2010). Anti-tuberculosis drug resistance in new and previously treated pulmonary tuberculosis cases in Burkina Faso. Int. J. Tuberc. Lung. Dis. 14(11):1424-1429.

WHO (2015). WHO policy statement: molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis. http://www.who.int/tb/laboratory/ line _ probe _assays/en/. WHO website.

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