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MINI-REVIEW

# Long-term preservation of *Leptospira* spp.: challenges and prospects

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## Abstract

Preservation of leptospiral cultures is tantamount to success in leptospiral diagnostics, research, and development of preventive strategies. Each *Leptospira* isolate has imperative value not only in disease diagnosis but also in epidemiology, virulence, pathogenesis, and drug development studies. As the number of circulating leptospires is continuously increasing and congruent with the importance to retain their original characteristics and properties, an efficient long-term preservation is critically needed to be well-established. However, the preservation of *Leptospira* is currently characterized by difficulties and conflicting results mainly due to the biological nature of this organism. Hence, this review seeks to describe the efforts in developing efficient preservation methods, to discover the challenges in preserving this organism and to identify the factors that can contribute to an effective long-term preservation of *Leptospira*. Through the enlightenment of the previous studies, a potentially effective method has been suggested. The article also attempts to evaluate novel strategies used in other industrial and biotechnological preservation efforts and consider their potential application to the conservation of *Leptospira* spp.

**Keywords** Preservation · Microorganism · Fastidious · *Leptospira*

## Introduction

Leptospires, the etiologic agent of leptospirosis, are important emerging bacterial pathogen causing a wide range of clinical illness in both humans and animals. The disease is responsible for high mortality in humans and susceptible animal species especially in endemic tropical regions with high rainfall. Leptospires are characterized by diversity in serovar distribution from region to region. To date, the genomospecies of the genus *Leptospira* is divided into 23 species. Phylogenetic analysis, based on 16S rRNA and whole-genome sequences, has grouped the 23 leptospires species into three monophyletic clusters: ten pathogenic species (*L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, *L. kmetyi*, *L. alstonii*, and *L. mayottensis*); six intermediate species *(L. inadai*, *L. fainei*, *L. broomii*, *L. wolffii*, *L. licerasiae*, and *L. venezuelensis*); and seven saprophytic species (*L. biflexa*, *L. wolbachii*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*, *L. idonii*, and *L. yanagawae*) (Bourhy et al. 2014; Puche et al. 2017). Currently, over 300 serovars of *Leptospira* spp. have been identified with more than 250 being pathogenic (Picardeau 2017; Thibeaux et al. 2018; Xu et al. 2017).

Each of the isolated and identified *Leptospira* serovars has important value in epidemiological understanding and disease diagnosis (Haake and Levett 2015). Furthermore, the current reference standard for the diagnosis of leptospiral infection, the microscopic agglutination test (MAT) also requires live leptospires as antigens (Musso and La Scola 2013). Thus, the continuous existence of the *Leptospira* isolates is of paramount importance. Besides, there is also the need to maintain the different strains in order to address many research questions that may arise from pathogenicity studies to vaccine development (Adler et al. 1986; Johnson and Rogers 1964; Myers and Varela-Diaz 1973). Currently, in comparison to other microorganisms, standard preservation protocols appear to be less successful for *Leptospira* spp. The common maintenance method entails a periodic sub-culture into fresh media either in liquid or semisolid Ellinghausen and McCullough modified by Johnson and Harris (EMJH) and Fletcher media

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at intervals of 3 to 12 weeks (Day and Stacey 2007; Faine et al. 1999; Rossetti and Auteri 2008; Samir and Wasfy 2013; Wuthiekanun et al. 2014). However, this preservation method is time-consuming and also associated with risk of infection due to the need of handling live cultures, potentials for contamination with fast-growing bacteria from the working environment as well as the possibility of cross-contamination among different strains and serovars. There is also a tendency for loss of virulence due to repeated passages. These outcomes can adversely affect both vaccine-related and diagnostic type of research due to change in the integrity and conformation of the surface exposed antigenic determinants. As a result, considerable efforts have been devoted to searching for efficient means of preserving and maintaining leptospiral cultures for long periods without any deleterious consequence on the viability and virulence of the bacteria.

This article attempts to provide an overview of the various methods employed for the long-term preservation of leptospires as well as their peculiar challenges. It also seeks to address the prospects and way forward with regard to the suitable methods to adopt especially in resource-limited endemic regions.

## Survival of leptospires

Both pathogenic and saprophytic leptospires essentially require the same kind of nutrients and temperature to survive. However, while saprophytic leptospires spend their entire life in soil and water albeit rarely found in humans and animals, pathogenic species require an animate object to complete their life cycles. The possession of the corkscrew-shape and periplasmic flagella are thought to aid the long-term survival of leptospires both within the host and in the environment (Mohammed et al. 2011). Nevertheless, survival and longevity of leptospires depend mainly on the moisture, pH, and temperature of the environment as well as essential nutrients required for growth (Barragan et al. 2017; Garba et al. 2017a). Leptospires are able to form biofilm with other microbes, and this mechanism is thought as one of the factors for their persistence and prolonged survival in the environment (Kumar et al. 2015). Despite being able to survive in the environment for longer periods, the ability of pathogenic leptospires to replicate outside their animate host is hampered. Instead, they exhibit limited instances of binary fission as an adaptation to long-term absence of suitable host species, or in other cases aggregating together to form a biofilm (Evangelista and Coburn 2010).

Nonetheless, the factors that contribute to the long-term survival, growth, and multiplication of leptospires in organs and tissues as well as in the environment is poorly understood. Continuous replenishment from the urine of infected animals may be responsible for their persistence in the environment, rather than long-term survival or binary fission as claimed by some authors (Garba et al. 2017b).

## Conventional methods for laboratory maintenance of leptospires

Leptospiral isolates are usually maintained in the laboratory by periodic sub-culturing in fresh media from the previous stock culture. As a routine practice, leptospires are maintained at room temperature either in the liquid or semisolid medium by sub-culturing into fresh medium every 3 to 12 weeks and the viability is monitored weekly by observing under a dark-field microscope (Faine et al. 1999). The culture medium, incubation temperature, shaking during incubation for aeration and nutrient availability, the passage time interval, and cultivation between passages are important for the efficient growth of leptospires. However, the frequent passage can also result in irreversible changes in strains due to the development of variants and mutants (Kumar et al. 2013). Nevertheless, this type of preservation is simple; it can pose significant risks to personnel due to frequent handling of live leptospires. Other factors bedeviling this method are as follows: high risk of cross-contamination between the cultures during sub-culturing, possible loss of strain or its virulence properties in pathogenic strains, long-term incubation and labor intensive as well as the requirement for biosafety level 2 facilities and skilled staff (Reed et al. 2000; Wuthiekanun et al. 2014).

## Medium for the cultivation of leptospires

Although simple, the nutritional requirement of leptospires is unique and specific. Up to date, only vitamin B1, vitamin B12, and long-chain fatty acids are known to be required. Several types of media have been described and developed for cultivation of leptospires. First is the traditional media: Noguchi, Korthof, Vervoort, Fletcher, and Stuart (Fletcher 1928; Korthof 1928; Noguchi 1918; Stuart 1946; Vervoort 1923). These media contain approximately 8–10% rabbit serum as a source of vitamin B12, which is necessary for the multiplication of leptospires. However, all these media are not widely used due to their low content of organic nitrogenous molecules, which is essential for the growth of leptospires (Czekalowski et al. 1954). Secondly and the most commonly used medium is the oleic-acid-albumin media of EMJH (Ellinghausen and McCullough 1965). EMJH is composed of a basic medium which includes disodium phosphate, monopotassium phosphate, ammonium chloride, and thiamine as well as enrichment factors such as albumin and Tween 80. EMJH medium is prepared either without antibiotic (non-selective) or with the addition of 5-fluorouracil and nalidixic acid (selective), which inhibit the growth of

microbial contaminants (Miraglia et al. 2009). EMJH medium can be prepared in liquid, semisolid, or solid form. In addition to the aforementioned, a low or protein-free medium has also been demonstrated to exhibit excellent cultural properties including retention of virulence and antigenicity of cultured pathogenic strains (Beyt and Johnson 1978). The medium is composed of polysorbates, vitamins B12 and B1, and inorganic salts. Due to these properties, it is considered suitable for culturing leptospires for application in the preparation of vaccines. Cultivation of leptospires requires the incubation of the culture in dark at 28–30 °C. Positive growth is usually observed after several weeks depending on the species and serovars, the source of culture, initial inoculum, and the nutrient composition of the media (Adler and Pena Moctezuma 2010; Levett 2001). Pathogenic species take longer time to grow than the non-pathogenic and intermediate strains (Cameron 2015). Sub-cultures from pure stock grow faster than primary isolation from the source (Levett 2001).

## Long-term preservation and storage methods for *Leptospira* spp.

### Freezing

Preservation by freezing is the simplest method of storing bacteria and other organisms (Kumar et al. 2013). Freezing temperature induces a state of dormancy within the cells that completely halt all cellular and metabolic activities without any adverse physiological and genetic alteration of the microorganisms (Coghlan et al. 1967). Prolonged storage of *Leptospira* by freezing has been demonstrated since 1939. Turner and Fleming showed that the viability of *L. icterohemorrhagiae* was retained after 5, 6, and 10 months of storage at − 78 °C in gallon insulated container partly filled with 95% alcohol (Turner and Fleming 1939). While the percentage of leptospires survived was not available, this study reported that a large proportion of the preserved leptospires exhibit active motility. The limitation of freezing method is the unavoidable occurrence of ice formation that will result in dehydration and denaturation of biomolecules leading to the death of the bacteria (Coghlan et al. 1967). To minimize the negative effects of freezing, cryoprotectants in the form of dimethyl sulfoxide or glycerol were tested. Coghlan et al. (1967) preserved leptospires in culture media containing 7.5% glycerol and stored at − 79 °C in dry ice cabinet for 27 months. Unfortunately, only about half (96) of 177 cultures resulted in satisfactory growth. This led them to investigate other factors that may play role in the long-term survival of leptospires such as the age of the culture when preserved and the quality of the sub-culture medium used (Coghlan et al. 1967). As observed in their study, leptospires cultures of 3, 4, and 5 weeks old did not recover after periods of 5–19 months of storage while growth was still observed in cultures of 5–7 days old. The size of the inoculum also plays an important role in isolation or recovery. For example, leptospires could be isolated from frozen kidneys of hamsters experimentally infected with $10^7$–$10^9$ cells but not from those infected with $10^3$–$10^4$ bacteria (Miller et al. 1990). This finding was acknowledged by Samir and Wasfy (2013) who showed the complete recovery of all tested serovars (*L. bataviae*, *L. canicola*, *L. grippotyphosa*, *L. icterohemorrhagiae*, *L. pomona*, *L. pyrogenes*) of 7–14 days old culture at a concentration of $3 \times 10^8$ cells/ml frozen (− 70 °C) in EMJH liquid media even after 20 months. The outcome of the above studies underscores the beneficial role of suitable media, age, and inoculum size of culture in the long-term preservation of leptospires.

### Liquid nitrogen

Preservation in liquid nitrogen has been a preferred method for long-term storage of *Leptospira* organism (Rossetti and Auteri 2008). The reports on its suitability in *Leptospira* research is dated almost half a century ago when Torney and Bordt (1969) observed the viability of three *Leptospira* strains (*L. canicola*, *L. icterohemorrhagiae*, *L. pomona*) that was maintained over 22-month period storage in liquid nitrogen with very minimal drops in viability titers (1 to 2 logs, base 10). Their initial effort was followed up by Stalheim 2 years later; where he demonstrated that the addition of 10% glycerol as cryoprotective agent results in minimal reduction in the viability titers of pomona vaccine stored in liquid nitrogen in comparison to the other cryoprotective agents such as ascorbic acid, dextrin, dimethyl sulfoxide, and albumin (Stalheim 1971). The protective activity of 10% glycerol was further demonstrated by Alexander et al. (1972) where the leptospires cultured in EMJH medium and Stuart medium and cryopreserved in liquid nitrogen were recovered after 16 months and 5 years of storage respectively. The effectiveness of dimethyl sulfoxide (2.5%) as the cryoprotective agent in the preservation of leptospires in liquid nitrogen tested a decade later showed complete viability of the test strains throughout the 22 months storage period (Palit et al. 1986). In contrary to the effective protection of glycerol reported by Stalheim (1971) and Alexander et al. (1972), this study showed glycerol as toxic to the leptospires. Reed et al. (2000) evaluated the effect of different concentrations of glycerol and dimethyl sulfoxide on the recovery of virulent strains of *L. canicola* and *L. icterohemorrhagiae*, affirmed that concentration above 5% prolongs the recovery time of the leptospires after storage in liquid nitrogen. Years later, a more standardized protocol for storage of leptospires in liquid nitrogen was described by Rossetti and Auteri (Rossetti and Auteri 2008). In this study, an inoculum of $10^9$ cells of 43 leptospires grown in EMJH medium for 7 days was rapidly frozen using 10% glycerol as

the protectant and stored in liquid nitrogen for 54 months. Although all isolates could be recovered after 6 months, the recovery rates dropped to 93 and 83% after 18 and 54 months. Despite these flaws, liquid nitrogen preservation is still considered the most viable choice for preserving leptospires.

### Preservation by drying

Preservation of *Leptospira* by freeze-drying or vacuum drying of liquid cultures showed varying results (Annear 1974; Otsuka and Manako 1961; Stavitsky 1945). Freeze-drying reported no growth of leptospires (Stavitsky 1945) and successful preservation up to only 2 years (Otsuka and Manako 1961), while by vacuum drying bacteria could be recovered even after 10 years of storage respectively Annear (1974). While it is not clear whether the recovery of leptospires was influenced by the technique of drying either from the liquid state (Annear 1974) or frozen state (Otsuka and Manako 1961), the storage condition after drying affects the viability. Both studies reported storage at 4 °C yielded good recovery compared to the storage at room temperature. Freeze-drying and vacuum drying undoubtedly offer a convenient storage and transport; however, for leptospires, concern for operator's risk of infection might limit the utility of these methods (Annear 1974).

### *Leptospira* Vanaporn Wuthiekanun agar

Despite the prospects and relative affordability of some of the preservation techniques for *Leptospira* described above, it is worthy to emphasize that their application is most suitable for established laboratories with advanced equipment and well-trained personnel. Unfortunately, most of the leptospirosis endemic countries are impoverished with a limited financial resource to conduct cutting-edge researches including long-term preservation of organisms for future use. As a result, efforts are being put to develop more affordable and cheap techniques that can be applicable in resource-poor settings. This search has led Wuthiekanun and colleagues to develop a relatively economical method of preservation of leptospires (Wuthiekanun et al. 2014). They showed that modification of EMJH medium from semisolid and liquid to solid medium was able to preserve leptospiral isolates for 12 months at room temperature with very minimal contamination (3%). This new medium was named as *Leptospira* Vanaporn Wuthiekanun (LVW) agar. The medium contained three core ingredients such as the *Leptospira* Medium Base EMJH, *Leptospira* Enrichment EMJH, and sodium pyruvate (Wuthiekanun et al. 2013). Sodium pyruvate was used to promote a faster growth of leptospires by reducing the extended lag periods of small inoculum during incubation (Johnson et al. 1973; Staneck et al. 1973). To the core components, bacteriological agar or Noble agar (a refined solidifying agar) and rabbit serum are added. The growth of *Leptospira* in EMJH solid media containing 1% Noble agar and 10% rabbit serum was faster (6.2 times higher rate) than the baseline condition (bacteriological agar with 3% rabbit serum) (Wuthiekanun et al. 2013). While a high percentage of rabbit serum had been associated with the stimulation of *Leptospira* growth, the contribution of Noble agar in the rapid growth of *Leptospira* is not clearly defined. However, in comparison to the bacteriological agar, Noble agar has a low percentage of calcium, ash, potassium, sodium, magnesium, sulfate and sulfur and it had been suggested that any of these variables might contribute to the growth of *Leptospira* (Wuthiekanun et al. 2013). LVW agar is a simple method for preservation of leptospires, yet not suitable for a long-term preservation as the dryness of agar was observed after the 12-month period (Wuthiekanun et al. 2014). Nonetheless, the method is simple and affordable and requires less time and human labor to accomplish.

## Biological characteristics of *Leptospira* as a major challenge

Although previous studies showed the practicability of preservation of leptospires either by freezing, liquid nitrogen, lyophilization, or storage in a solid medium, the different and inconsistent protocol leads to the difficulty in the standardization of effective method and hard to be implemented as a routine practice. Despite the efforts to develop a standardized protocol for the preservation of leptospires, it needs to be understood that the inherent biologic characteristics of the organism itself contribute to a large degree on the success of preservation. Leptospires are motile spirochetes that can move individually. Motile organisms have been reported to have lower survival rate after preservation by lyophilization (Miyamoto-Shinohara et al. 2008). Although poorly explained, the possible reason for the low survival rate of motile organisms compared to the non-motile bacteria could be the higher exposure to the ice formed surrounding the cells during freezing. Non-motile bacteria usually form cluster and this can help the bacteria protecting each other from the detrimental effect of the surrounding ice. The mechanism of cell aggregation as a survival method of leptospires was observed by Trueba et al. (2004). In their study, the *Leptospira* that was incubated in a viscous environment (a semisolid medium composed of distilled water and 0.5% purified agarose) produced cell aggregations and the survival of the *Leptospira* increased more than threefold (347 days) compared to free-swimming incubated in distilled water (110 days). The mechanism of cell aggregation may help the bacteria to endure harsh condition, either in low temperature or in a nutrient-poor environment by accumulating enzymes and nutrients from the lysing cells (Blat and Eisenbach 1995; Crespi 2001). This leptospiral aggregation property may also explain

why leptospires survive longer in a semisolid medium than in liquid medium.

The membrane structure of leptospires shares both of the Gram-negative and Gram-positive bacteria. The membrane of leptospires is homologous to Gram-negative by having a double-membrane and presence of lipopolysaccharide (LPS), while the close association of the peptidoglycan cell wall with the cytoplasmic membrane is a resemblance of the Gram-positive envelope architecture (Mohammed et al. 2011). However, similar to other Gram-negative bacteria, *Leptospira* has a thin peptidoglycan cell wall. The thin cell wall has a greater tendency to rupture when exposed to the harsh environment during freezing, desiccation, and rehydration (Miyamoto-Shinohara et al. 2008). The thickness of cell walls undoubtedly influences the survival rate of bacteria as observed by Miyamoto-Shinohara et al. (2008), where Gram-positive bacteria exhibited higher survival rate than the Gram-negative bacteria after preservation by freeze-drying.

Another feature of *Leptospira* spp. is the presence of LPS. LPS helps bacteria to survive in the desiccant environment by retaining water in the cells (Billi and Potts 2002; Potts 1994; Schnider-Keel et al. 2001). During freezing and lyophilization, a complete removal of water from the cell is required to prevent the formation of ice inside the cells and occurrence of moisture during storage of dried culture. The LPS prevents the removal of water molecules from inside the cells during freeze-drying (Miyamoto-Shinohara et al. 2008).

The fastidious nature of the *Leptospira* spp. challenges their primary isolation, routine propagation, and storage (Zuerner, 2005). Generally, for fastidious microorganisms, preservation by lyophilization is less successful and cryopreservation is usually useful only for the short-term period (Hoefman et al. 2012). This explains the reason for the critical need of periodic sub-culture for maintenance of many fastidious microorganisms including leptospires.

## Toward a more standardized preservation method

Due to the large number of *Leptospira* isolates, preservation by freezing and liquid nitrogen offers a more efficient method of storing leptospires as it saves storage space and time as well as a reduction in workload and risk of infection to the operators in a routine sub-culture. The success of preservation either by freezing or in liquid nitrogen depends on several factors and each of these factors is interdependent. These factors include age and concentration of organisms, freezing and thawing rate as well as the type of cryoprotectants. The culture at log-phase, from 4 to 14 days with a concentration of more than $3 \times 10^8$ cells per milliliter showed a high survival rate (Rossetti and Auteri 2008; Samir and Wasfy 2013). A rapid cooling showed a more consistent recovery compared to slow cooling (Alexander et al. 1972; Coghlan et al. 1967; Rossetti and Auteri 2008). Glycerol and dimethyl sulfoxide at a concentration of 10% or lower showed a better protective activity than the concentration of more than 10% (Reed et al. 2000). An optimized condition for all these factors showed full recovery for a period of 6, 16, and 20 months (Alexander et al. 1972; Rossetti and Auteri 2008; Samir and Wasfy 2013). However, extended storage period showed a consistent reduction in the recovery of leptospires (Alexander et al. 1972; Rossetti and Auteri 2008). This occurrence suggested that apart from injury during freezing and thawing process, there is an appreciable, continuous damage during the storage period which will eventually lead to the death of the organisms. Despite the mechanism is poorly understood, earlier studies have shown that the viability and recovery of microorganisms are reduced in an extended storage period at low temperature (Reed et al. 2000; Straka and Stokes 1959). As shown in Fig. 1, there are three states of cells after freezing, which are non-injured, sub-lethally injured, and killed. The sub-lethally injured cells are grouped into two: structurally and metabolically injured. Although preserved in optimized conditions, it will never be possible to eliminate the occurrence of injury and death of organisms while freezing, during the storage and thawing. More attention should be given to the sub-lethally injured cells, as these cells are still viable and can be recovered if grown in an appropriate and suitable condition (Straka and Stokes 1959; Wu 2008). The structurally injured cells are prone to death due to the inhibitory substances that are present in the selective medium, hence need to be cultured first on non-selective medium to allow repair before their selective enumeration. The damage in structurally injured cells can be more extensive as the metabolic systems are damaged thus requiring more complex medium to support their growth. Studies on structural and metabolic injury in other microorganisms such as *Shigella sonnei* and *Escherichia coli* have shown to grow well in non-selective medium and required a more complex medium for recovery (Nakamura and Dawson 1962; Ray and Speck 1972). For leptospires, in addition to the factors listed above, a modification in medium composition to sub-culture the leptospires after storage at low temperature is suggested for a better recovery of the cells. Although actively motile upon thawing after long-term storage in liquid nitrogen, recovery rates are lower during sub-culturing. This could be overcome by the addition of growth factors in the sub-culture media that stimulate the multiplication of the organisms devitalized during the freezing process (Coghlan et al. 1967). An enriched and non-selective medium is suggested to allow the repair of the injured leptospires before being enumerated in selective medium. Repair methods also include the addition of compounds that contributes in the resynthesizing of cellular components or removal of inhibitors that may affect the mechanism of repair. For example, the presence of hydrogen peroxide ($H_2O_2$) produced in culture media is toxic to the

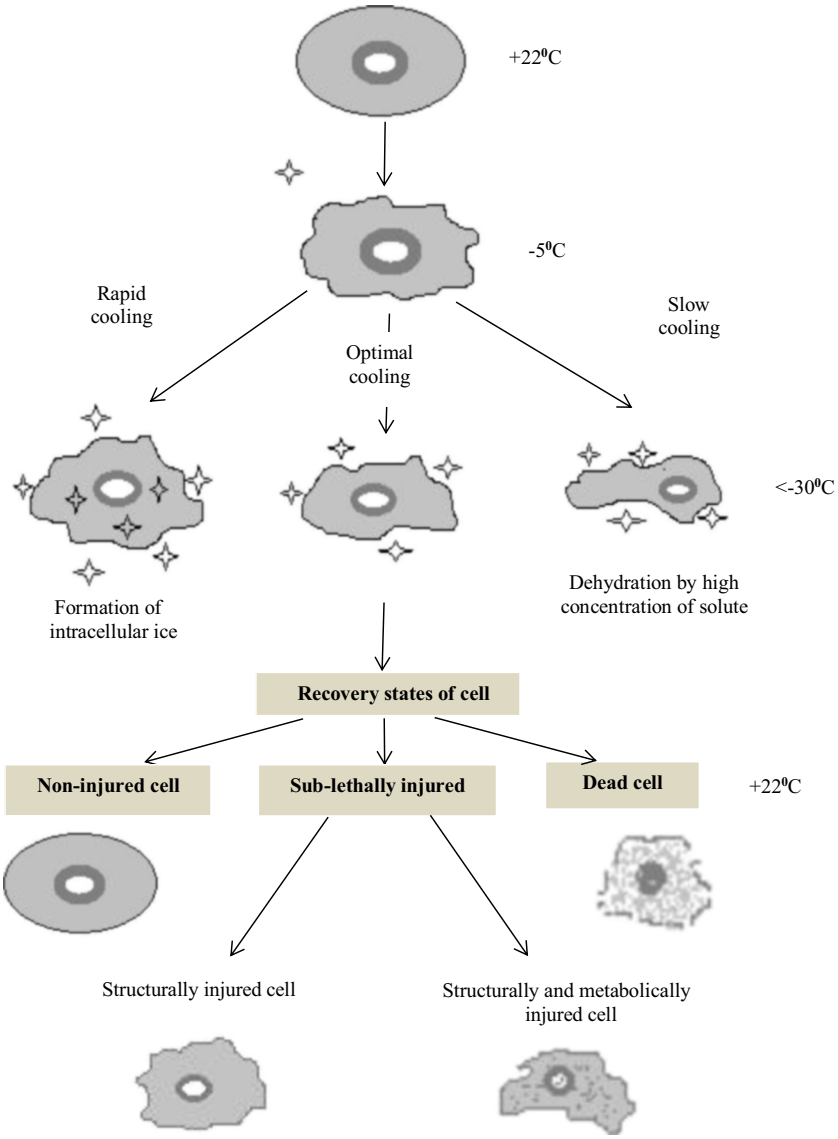


**Fig. 1** Cell exposed to low temperature subjected to damage by formation of ice and dehydration. The occurrence of both effects depends on the cooling rate and can be prevented by addition of cryoprotectants. However, successful freezing still does not eliminate the possibility of injury to the cells due to the prolonged storage in low temperature

injured leptospires and this product could be removed by the addition of compounds that decompose $H_2O_2$ such as the enzyme catalase or pyruvate (Staneck et al. 1973). To date, very limited studies are available on the media composition for sub-culture or retrieval post preservation. Hence, we emphasize to evaluate more sub-culture media compositions for complete retrieval of cells, which is very important for the effective long-term preservation of leptospires.

## Prospects and emerging preservation techniques

Unlike plants and animal species, little attention has been devoted to the conservation of microbial diversity despite their enormous benefits and applications in biological processes, industrial revolutions, and preservation of the ecosystems (Cockell and Jones 2009). Recent investigations in the long-term survival of leptospires based on bioinformatic, genetic, and biochemical evidence has demonstrated the presence of the EbpA-RpoN pathway whereby *Leptospira* bacterial enhancer-binding protein EbpA and the sigma factor 54 interact synergistically to activate a number of genes that play important role in the long-term survival of *L. interrogans* (Hu et al. 2016). However, due to the lack of robust tools for genetic manipulation of this spirochete, knowledge about their long-term survival is still vague. The accurate estimation of survival time and preservation of virulence by pathogenic *Leptospira* spp. has equally become crucial in the determination of the environmental risk it poses upon excretion in the environment as well as the adoption of appropriate preventive measures (Thibeaux et al. 2017). As highlighted earlier, long-term preservation in artificial media may depend on their genetic heterogeneity which still remains to be unraveled.

Due to the importance of long-term preservation of these microbial populations, newer technologies have emerged in part to complement the already established protocols. These include the following: microencapsulation using novel high-voltage electrohydrodynamic processes like electrospinning and electrospraying which permits the shielding of the cells thereby protecting it from the adverse effect of freezing, at the same time increasing the stability of the cell during storage. Microencapsulation shields biological cells against adverse surrounding environment which destruct their core. It protects the bacteria from heat, oxygen, and moisture as well as pH fluctuations for which leptospires are known to be susceptible (Kala et al. 2014). The microcapsules are usually formed using natural materials such as sugar, gums, protein, lipid, and synthetic or modified polymers, which are used to formulate gel beads. Microencapsulation has proven to be useful in the development and delivery of *Leptospira* vaccine antigens (Inanlou et al. 2015). Several particulate antigens have been embedded in biodegradable polymers like poly-lactic-coglycolic acid (PLGA), poly-L-lactid (PLLA) and alginate and used for immunization studies with the outcome indicating promising results (Faisal et al. 2009). This technology has also shown promise particularly in the probiotics field, where it serves to provide a controlled release of probiotic cells in the human gut under favorable conditions (Aldabran et al. 2015).

In addition, cellular immobilization during cultivation as against the harsh effect of freezing and drying that can result in irreversible denaturation of membrane proteins and other cellular components of the organism can be viewed as a good alternative, especially as it can lead to increase the stability of the microorganisms (Carvalho et al. 2004). It entails the retention of viable cells in a discrete spatial location in order to achieve high cell concentration. The technique has been shown to be useful in the biomass and metabolite production in comparison with free cell systems (Flickinger et al. 2010). Cell immobilization is regarded as a convenient method due to its low cost and improved stability. Successfully immobilized cells have been used in the areas of biotechnology and industrial production (Elakkiya et al. 2016). It is regarded as a novel microbial preservation technique that permits entrapment of cells in water-soluble polymers (acacia gum or pullulan) via a polymerization and water replacement process that results in the formation of a protective stable film which confers protection to the microorganisms during exposure to unfavorable conditions (Krummow et al. 2009; Sorokulova et al. 2015). In this way, the polymer traps the water, thereby preventing the complete dehydration of the cell cytoplasm, maintaining the water balance of live cells and also increasing their viability (Krummow et al. 2009). Furthermore, the application of sub-lethal stresses as simple and effective strategies to improve cellular properties while preserving the cellular functionality during subsequent processing and storage has recently been given due consideration (Alonso 2016). An essential component of successful preservation protocol is to ensure cellular robustness. The application of sub-lethal stress bioprocessing strategies has lately emerged as an effective approach to enhance cellular robustness during cultivation and prior to downstream processing.

The major concerns in preservation of leptospires should be to develop affordable and less labor-intensive protocols, optimize the EMJH media for longer storage of leptospires, develop suitable cryoprotectants and unraveling the ambiguous molecular mechanisms responsible for the induction of morphological, physiological, and genetic alterations that occur during preservation (Prakash et al. 2013; Trivedi et al. 2012). Moreover, research on the effect of different preservation techniques to different phases of the cultures as well as the optimization of leptospiral densities for preservation is necessary in order to provide and achieve more viability. In this regard, development of alternative strategies rather than the established conventional methods is essential so as to reduce the cost of preservation, extend the shelf-life of the bacteria, and provide less expensive storage especially in resource-poor settings (Trivedi et al. 2012).

## Conclusion

The need to sustain and preserve microbes while retaining their cellular viability still remains an elution. The preservation of leptospires is critical for ensuring the existence of the important strains and for future research. Novel methods, with the ability to retain leptospiral viability as well as the stability of the genetic material after long-term storage, will immensely benefit the scientific community. Development of easy, convenient, and yet still conserving the viability, physiology, morphology, and genetics of pure strains as well as the ability to allow the maximum recovery after storage is paramount. However, it is also important to preserve the leptospires in as many as different forms possible to save these delicate organisms.

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### Compliance with ethical standards

**Ethical statement** This study does not contain any human participants or animals, hence do not require ethics approval.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References


Adler B, de la Pena Moctezuma A (2010) *Leptospira* and leptospirosis. Vet Microbiol 140(3–4):287–296. https://doi.org/10.1016/j.vetmic.2009.03.012

Adler B, Faine S, Christopher WL, Chappel RJ (1986) Development of an improved selective medium for isolation of leptospires from clinical material. Vet Microbiol 12(4):377–381

Aldabran HA, Chatzifragkou A, Khutoryanskiy VV, Charalampopoulos D (2015) Stability of probiotic *Lactobacillus plantarum* in dry microcapsules under accelerated storage conditions. Food Res Int 74: 2018–2216. https://doi.org/10.1016/j.foodres.2015.05.016

Alexander AD, Lessel EF, Evans LB, Franck E, Green SS (1972) Preservation of leptospires by liquid-nitrogen refrigeration. Int J Syst Bacteriol 22(3):165–169

Alonso S (2016) Novel preservation techniques for microbial cultures. In: Ojha KS, Tiwari BK (eds) Novel food fermentation technologies. Springer International Publishing, Switzerland, pp 7–33

Annear DI (1974) Recovery of leptospires after dry storage for ten years. Int J Syst Bacteriol 24(4):399–401

Barragan V, Olivas S, Keim P, Pearson T (2017) Critical knowledge gaps in our understanding of environmental cycling and transmission of *Leptospira* spp. Appl Environ Microbiol 83(19):e01190–e01117. https://doi.org/10.1128/AEM.01190-17

Beyt RF, Johnson RC (1978) Protein-free and low-protein media for the cultivation of *Leptospira*. Infect Immun 19(2):562–569

Billi D, Potts M (2002) Life and death of dried prokaryotes. Res Microbiol 153(1):7–12

Blat Y, Eisenbach M (1995) Tar-dependent and -independent pattern formation by *Salmonella typhimurium*. J Bacteriol 177(7):1683–1691

Bourhy P, Collet L, Brisse S, Picardeau M (2014) *Leptospira mayottensis* sp. nov., a pathogenic species of the genus *Leptospira* isolated from humans. Int J Syst Evol Microbiol 64(12):4061–4067. https://doi.org/10.1099/ijs.0.066597-0

Cameron CE (2015) Leptospiral structure, physiology, and metabolism. Curr Top Microbiol Immunol 387:21–41. https://doi.org/10.1007/978-3-662-45059-8_3

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2004) Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. Biotechnol Prog 20: 248–254. https://doi.org/10.1021/bp034165y

Cockell CS, Jones HL (2009) Advancing the case for microbial conservation. Oryx 43(4):520–526

Coghlan JD, Lumsden WH, McNeillage GJ (1967) Low-temperature preservation of *Leptospira*, preliminary communication. J Hyg (Lond) 65(3):373–379

Crespi BJ (2001) The evolution of social behavior in microorganisms. Trends Ecol Evol 16(4):178–183

Czekalowski JW, McLeod JW, Rodican J (1954) Observations on the nutrition of *Leptospira* based on the development of Dinger's phenomenon. J Gen Microbiol 10(2):199–208. https://doi.org/10.1099/00221287-10-2-199

Day JG, Stacey G (2007) Cryopreservation and freeze-drying protocols, 2nd edn. Humana Press, Totowa

Ellinghausen HC, McCullough WG (1965) Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex (OAC) and a medium of bovine albumin and polysorbate 80. Am J Vet Res 26:45–51

Elakkiya M, Prabhakaran D, Thirumarimurugan M (2016) Methods of cell immobilization and its applications. IJIRSET 5(4):5429–5433

Evangelista KV, Coburn J (2010) *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune response. Future Microbiol 5(9):1413–1425. https://doi.org/10.2217/fmb.10.102

Faine S, Adler B, Bolin C, Perolat P (1999) *Leptospira* and leptospirosis. CRC Press Inc, Melbourne

Faisal SM, Yan W, McDonough SP, Chang CF, Pan MJ, Chang YF (2009) Leptosome-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model. Vaccine 27(47):6537–6545. https://doi.org/10.1016/j.vaccine.2009.08.051

Fletcher W (1928) Recent work on leptospirosis, tsutsugamushi disease, and tropical typhus in the Federated Malay States. Trans R Soc Trop Med Hyg 21(4):265–282

Flickinger MC, Verica M, Branko B, Viktor N (2010) Immobilized cells. In: Flickinger MC (eds) Encyclopedia of industrial biotechnology. https://doi.org/10.1002/9780470054581.eib365

Garba B, Bahaman AR, Bejo SK, Zakaria Z, Mutalib AR (2017a) Retrospective study of leptospirosis in Malaysia. EcoHealth 14: 389–398. https://doi.org/10.1007/s10393-017-1234-0

Garba B, Bahaman AR, Bejo SK, Zakaria Z, Mutalib AR, Bande F (2017b) Major epidemiological factors associated with leptospirosis in Malaysia. Acta Trop 178:242–247. https://doi.org/10.1016/j.actatropica.2017.12.010

Johnson RC, Rogers P (1964) 5-Fluorouracil as a selective agent for the growth of leptospirae. J Bacteriol 87(2):422–426

Johnson RC, Walby J, Henry RA, Auran NE (1973) Cultivation of parasitic leptospires: effect of pyruvate. Appl Microbiol 26(1):118–119

Haake DA, Levett PN (2015) Leptospirosis in humans. Curr Top Microbiol Immunol 387:65–97. https://doi.org/10.1007/978-3-662-45059-8_5

Hoefman S, Van Hoorde K, Boon N, Vandamme P, De Vos P, Heylen K (2012) Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS One 7(14):e34196. https://doi.org/10.1371/journal.pone.0034196

Hu WL, Pappas CJ, Zhang JJ, Yang YY, Yan J, Picardeau M, Yang MX (2016) The EbpA-RpoN regulatory pathway of the pathogen *Leptospira interrogans* is essential for survival in the environment. App Environ Microbiol 83(3):e02377–e02316. https://doi.org/10.1128/AEM.02377-16

Inanlou F, Khaki P, Mohammadpour N, Bidhendi SM, Zolfagharian H, Chang YF, Bazrafshan MR, Saraei F (2015) Preparation of microspheres containing leptospiral antigen using biodegradable alginate polymers. Vet Arh 85(4):395–406

Kala H, Mer KKKS, Kumar S (2014) A review on mechanical and tribological behaviors of stir cast aluminium matrix composites. Procedia Mater Sci 6:1951–1960

Korthof G (1928) Proeven met *Leptospira icterohaemorrhagiae*. Versl Volksgexondh.Amsterdam, p 1272

Krummow AA, Sorokulova IB, Olsen E, Globa L, Barbaree JM, Vodyanoy VJ (2009) Preservation of bacteria in natural polymers. J Microbiol Methods 78(2):189–194. https://doi.org/10.1016/j.mimet.2009.05.017

Kumar S, Kashyap PL, Singh R, Srivastava AK (2013) Preservation and maintenance of microbial cultures. In: Arora D, Das S, Sukumar M (eds) Analyzing microbes: manuals of molecular biology techniques. Springer Protocols Handbooks. Springer, Berlin, Heidelberg, pp 135–152

Kumar KV, Lall C, Raj RV, Vedhagiri K, Vijayachari P (2015) Coexistence and survival of pathogenic leptospires by formation of biofilm with *Azospirillum*. FEMS Microbiol Ecol 91(6).pii: fiv051. https://doi.org/10.1093/femsec/fiv051

Levett PN (2001) Leptospirosis. Clin Microbiol Rev 14(2):296–326. https://doi.org/10.1128/CMR.14.2.296-326.2001

Miller DA, Wilson MA, Beran GW (1990) The effect of storage time on isolation of *Leptospira interrogans* from bovine kidneys. J Vet Diagn Invest 2(1):63–65. https://doi.org/10.1177/104063879000200112

Miraglia F, de Moraes ZM, Melville PA, Dias RA, Vasconcellos SA (2009) EMJH medium with 5-fluorouracil and nalidixic acid associated with serial dilution technique used to recover *Leptospira* spp from experimentally contaminated bovine semen. Braz J Microbiol 40(1):189–193. https://doi.org/10.1590/S151783822009000100033

Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, Nakahara T (2008) Survival of freeze-dried bacteria. J Gen Appl Microbiol 54(1):9–24

Mohammed H, Nozha C, Hakim K, Abdelaziz F, Rekia B (2011) *Leptospira*: morphology, classification, and pathogenesis. J Bacteriol Parasitol 2:6–9

Musso D, La Scola B (2013) Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect 46(4):245–252. https://doi.org/10.1016/j.jmii.2013.03.001

Myers DM, Varela-Diaz VM (1973) Selective isolation of leptospires from contaminated material by incorporation of neomycin to culture media. Appl Microbiol 25(5):781–786

Nakamura M, Dawson DA (1962) Role of suspending and recovery media in the survival of frozen *Shigella sonnei*. Appl Microbial 10:40–43

Noguchi H (1918) Further study on the cultural conditions of *Leptospira* (*Spirochaeta*) *icterohaemorrhagiae*. J Exp Med 27(5):598–608

Otsuka S, Manako K (1961) Studies on the preservation of leptospirae by freeze-drying. Jpn J Microbiol 5:141–148

Palit A, Haylock LM, Cox JC (1986) Storage of pathogenic leptospires in liquid nitrogen. J App Bacteriol 61(5):407–411

Picardeau M (2017) Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat Rev Microbiol 15(5):297–307. https://doi.org/10.1038/nrmicro.2017.5

Potts M (1994) Desiccation tolerance of prokaryotes. Microbiol Rev 58(4):755–805

Prakash O, Nimonkar Y, Shouche YS (2013) Practice and prospects of microbial preservation. FEMS Microbiol Lett 339(1):1–9. https://doi.org/10.1111/1574-6968.12034

Puche R, Ferres I, Caraballo L, Rangel Y, Picardeau M, Takiff H, Iraola G (2017) *Leptospira venezuelensis* sp.nov., a new member of the intermediate group isolated from rodents, cattle and humans. Int J Syst Evol Microbiol 68(2):513–517. https://doi.org/10.1099/ijsem.0.002528

Ray B, Speck ML (1972) Repair of injury by freezing *Escherichia coli* as influenced by recovery medium. Appl Microbiol 24(2):258–263

Reed NE, Varney WC, Goddard RD, Wyeth PJ (2000) The maintenance of challenge strains used in the potency test for canine *Leptospira* vaccines. Biologicals 28(1):25–28. https://doi.org/10.1006/biol.1999.0235

Rossetti CA, Auteri CD (2008) Long-term preservation of leptospires by liquid nitrogen. Rev Argent Microbiol 40(2):86–88

Samir A, Wasfy MO (2013) A simple technique for long-term preservation of leptospires. J Basic Microbiol 53(3):299–301. https://doi.org/10.1002/jobm.201100551

Schnider-Keel U, Lehbolle KB, Baehler E, Haas D, Keel C (2001) The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol 67(12):5683-5693. https://doi.org/10.1128/AEM.67.12.5683-5693.2001

Sorokulova I, Olsen E, Vodyanoy V (2015) Biopolymers for sample collection, protection, and preservation. App Microbiol Biotechnol 99:5397–5406

Stuart RD (1946) The preparation and use of a simple culture medium for leptospirae. J Path Bact 58(3):343–349

Stalheim OH (1971) Viable, avirulent *Leptospira interrogans* serotype Pomona vaccine: preservation in liquid nitrogen. Appl Microbiol 22(4):726–727

Staneck JL, Henneberry RC, Cox CD (1973) Growth requirements of pathogenic *Leptospira*. Infect Immun 7(6):886–897

Stavitsky AB (1945) Preservation of *Leptospira Icterohemorrhagiae* in vitro. J Bacteriol 50(1):118–119

Straka RP, Stokes JL (1959) Metabolic injury to bacteria at low temperatures. J Bacteriol 78(2):181–185

Thibeaux R, Iraola G, Ferres I, Bierque E, Girault D, Saupe-Gilbert M, Picardeaue M, Goarant C (2018) Deciphering the unexplored *Leptospira* diversity from soils uncovers genomic evolution to virulence. Microbial Genomic 4(1):e000144. https://doi.org/10.1099/mgen.0.000144

Thibeaux R, Geroult S, Benezech C, Chabaud S, Soupé-Gilbert ME, Girault D, Bourque E, Goarant C (2017) Seeking the environmental source of leptospirosis reveals durable bacterial viability in river soils. PLoS Negl Trop Dis 11(2):e0005414. https://doi.org/10.1371/journal.pntd.0005414

Torney HL, Bordt DE (1969) Viability quantitation of leptospires after rapid and controlled rate freezing. Cryobiology 5(5):352–254

Trivedi P, Pandey A, Lok Man SP (2012) Bacterial inoculants for field applications under mountain ecosystem: present initiatives and future prospects. In: Maheshwari DK (ed) Bacteria in agrobiology: plant probiotics. Springer-Verlag, Berlin, pp 15–44

Trueba G, Zapata S, Madrid K, Cullen P, Haake D (2004) Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. Int Microbiol 7(1):35–40

Turner TB, Fleming WL (1939) Prolonged maintenance of spirochetes and filterable viruses in the frozen state. J Exp Med 70(6):629–637

Vervoort H (1923) Spirochaeten bij acute koortsige ziekten ven onbekenden oorsprong in de tropen. Geneesk Tijdschr Ned Ind 63:800

Wu VC (2008) A review of microbial injury and recovery methods in food. Food Microbiol 25(6):735–744. https://doi.org/10.1016/j.fm.2008.04.011

Wuthiekanun V, Amornchai P, Paris DH, Langla S, Thaipadunpanit J, Chierakul W, Smythe LD, White NJ, Day NPJ, Limmathurotsakul D, Peacock SJ (2013) Rapid isolation and susceptibility testing of *Leptospira* spp. using a new solid medium, LVW agar. Antimicrob Agents Chemother 57(1):297–302. https://doi.org/10.1128/AAC.01812-12

Wuthiekanun V, Amornchai P, Langla S, Oyuchua M, Day NPJ, Limmathurotsakul D (2014) Maintenance of *Leptospira* Species in *Leptospira* Vanaporn Wuthiekanun Agar. J Clin Microbiol 52(12):4350–4352. https://doi.org/10.1128/JCM.02273-14

Xu Y, Zheng H, Zhang Y, Wang Y, Zhang J, Li Z, Cui S, Xin X, Ye Q, Chang YF, Wang J (2017) Genomic analysis of new serovar of *Leptospira weilii* serogroup Manhao. Front Microbiol 8:149. https://doi.org/10.3389/fmicb.2017.00149

Zuerner RL (2005) Laboratory maintenance of pathogenic *Leptospira*. Curr Protoc Microbiol 12:Unit 12E.1. https://doi.org/10.1002/9780471729259.mc12e01s00