IMPACT OF GLYCEROL AND DIMETHYL SULFOXIDE ON THE PERSISTENCE OF PASTEURLELLA MULTOCIDA DURING ITS PRESEVATION AT DIFFERENT TEMPERATURES

W. Shahzad1, S. Hussain2, S. Hussain1, A. A. Nasir1, N. Mustafa1, A. Kausar1, H. Afroz1, S. Ali3 and S. Sattar1

1Veterinary Research Institute, Lahore Cantt, Pakistan
2Director, Veterinary Research Institute, Lahore
3Foot & Mouth Disease Research Centre, Lahore Cantt. Pakistan
*Corresponding author Email: waseem1971@hotmail.com

ABSTRACT: Bacterium Pasteurella multocida type B:2 is the causative agent of Hemorrhagic Septicemia in bovines in Pakistan. This organism is also being used for oil adjuvanted (Montanide ISA 50 V2) vaccine for mass vaccination in cattle & buffaloes in Punjab, Pakistan. A study was conducted to study the impact of glycerol and dimethyl sulfoxide (DMSO) on persistence of P. multocida during its course of storage at 0, 4 and – 20 °C. Pure seed of P. multocida was grown in Brain Heart Infusion (BHI) broth at pH 7.4 and incubated at 37 °C for 18 hrs. in shaking incubator. The growth was observed for its purity microscopically as well as culturing on MacConkey’s & Nutrient agar slants. The pure culture was divided into three parts. One part was added with 5 % sterilized DMSO (final concentration) and second part was added with 10 % sterilized DMSO (final concentration) and 15 % glycerine (final concentration) while third part was kept as control without addition of DMSO and glycerine. All three parts were aliquoted (1 mL) in sterilized 1.5 mL eppendroff tubes and stored at three different temperatures i.e, 0, 4 and – 20 °C. Eppendroff tubes from different temperatures were thawed in water bath (22 °C) and subjected to direct culturing on BHI broth, microscopic examination, purity testing on slants, culture turbidity / Optical Density (OD) values, dry biomass calculation and mice inoculation test for three months with 15 days intervals. The results indicated that pure P. multocida culture mixed with 10 % sterilized DMSO and 15 % sterilized glycerol stored at – 20 °C survived for three months and can be used for HS vaccine production without losing its survival.

Keywords: Haemorrhagic Septicaemia, Pasteurella multocida, Vaccine, DMSO, Glycerine

(Received 15.12.2021 Accepted 28.02.2022)

INTRODUCTION

Hemorrhagic Septicemia (HS) is the most lethal and economically significant disease of cattle and water buffalo in Asia, Africa and Middle East with its more incidences in South East Asia including Pakistan (Shahzad et al., 2013; O.I.E., 2018; Almoheer et al. 2022). Different surveillance studies have shown that Pasteurella multocida serotype B:2 causes high mortality (upto 50 %) in many areas of Pakistan (Khan et al., 2011). Buffalo calves having age of 6-12 months are more susceptible to this disease and high mortality has been observed in this age group (Farooq et al., 2011). The disease mostly occurs in rainy season. The environment (hot humid temperature), management (congestion, insufficient ventilation and transport) and malnourishment are among the probable reasons which are implicated to increase the incidence of HS disease in Pakistan (Tariq et al., 1997). Sudden onset and short duration of the disease symptoms are the main causes of treatment failure in affected animals. The disease can only be prevented through mass immunization agenda. Different varieties of vaccine preparations are available in Pakistan for prevention and mass vaccination of HS in bovines. These vaccines include alum, aluminium hydroxide gel based and oil based (Montanide ISA-50 V2) and P. multocida type B:2 is being used for preparation of these types of vaccines. Many workers have studied the survival of P. multocida at different temperatures (Watko and Heddleston, 1966; Thomson et al. 1992; Shah et al. 2008).

Cryopreservation is basically the storage of biological materials at very low temperatures so as to conserve their viability and it has become an important technique in most experimental and clinical protocols including bacterial preservation for vaccine development (Xie et al.2022). Biclot et al. (2022) claimed that the method of preservation has an important effect on the recovery and isolation of high and low abundant bacterial taxa further different preservation methods are needed to recover specific sets of taxa.

This research was conducted to study the influence of glycerol and Di Methyl Sulfoxide (DMSO) on survival of P. multocida during its storage at 0, 4 and – 20 °C.
MATERIALS AND METHODS

Working seed of Pasteurella multocida: Reconstitution of Lyophilized seed of Pasteurella multocida serotype B:2 was carried out with Nutrient broth and incubation was given at 37°C for 4-5 hours. Later on this reconstituted seed was inoculated sub-cutaneously in Swiss Albino mice and kept under observation for 24 hours. Almost after 18-20 hours, immediately after the death of mice, heart blood was collected aseptically after postmortem and cultured on Brain Heart Infusion (BHI) broth and incubation was given at 37 °C for 24 hours. Heart blood was also cultured on Nutrient, MacConkey’s, Sabouraud’s agar and thioglycolate media to check the purity and sterility of the seed. After 24 hours, clearing the purity microscopically and on the basis of growth features, the cultured seed in BHI was stored at 4 °C (O.I.E., 2018).

Media preparation: BHI broth (Merck, India) was prepared with concluding pH 7.4 for the cultivation of P. multocida. The growth medium was made sterile after incubating at 37 °C for 24 hours.

Sterile BHI broth was cultured with the pure seed of P. multocida serotype B:2 @ 5% (O.I.E., 2018).

Aeration process: Aeration was given to the culture by syringe filters (0.2 µm pore size, Maxipore, England) through a compressor and air was dispersed through a sparger. The vessels were incubated at 37 °C for 15-18 hours on a shaker at 60-80 rpm. The purity of growth was checked microscopically and sterility was observed on Nutrient agar, MacConkey’s agar, Sabouraud’s agar, Thioglycolate medium and Nutrient broth (O.I.E., 2018).

The pure culture was then divided into three parts. One part (A) was added with 5% sterilized DMSO (final concentration) and second part (B) was added with 10% sterilized DMSO (final concentration) and 15% glycerine (final concentration) while third part (C) was kept as control without addition of DMSO and glycerine. All three parts were aliquoted (1 mL) in sterilized 1.5 mL eppendorff tubes and stored at three different temperatures i.e. 0, 4 and – 20 °C.

Eppendorff tubes from different temperatures were thawed in water bath (22 °C) for 10-20 mints and subjected to direct culturing on BHI broth, microscopic examination (O.I.E., 2018), purity testing on slants (O.I.E., 2018), culture turbidity / Optical Density (OD) values, dry biomass calculation (Bratbak and Dundas, 1984) and mice inoculation test (O.I.E., 2018) for three months with 15 days intervals.

RESULTS AND DISCUSSION

Hemorrhagic Septicemia (HS) is a seasonal fatal disease of bovines and is prevented by mass vaccination schemes before rainy season in Punjab, Pakistan as the best measure to control the disease is vaccination (Almoheer et al., 2022). Immune response in vaccinated animals is mandatory against outer coat of P. multocida. (Bain et al., 1982). This outer coat is made up of lipopolysaccharide (LPS) and some parts of protein. This LPS is responsible for the generation of B lymphocytes response and cannot be taken up with Major Histocompatibility Complex class II (MHC- class II) antigen by Antigen Presenting Cells (APC) of the host animal body, and therefore the responsive B lymphocytes cannot obtain cooperation of T lymphocytes for enhanced antibody manufacturing. The reaction of B lymphocytes to LPS is crucial and the nature of protection is truncated and small duration (Abbas et al., 1991).

The oil based vaccine (OVB) forms depot of vaccine and its slow release over long duration, protect the antigen from rapid deterioration by enzymes and invites the APCs at the injection site and enhances the antigen uptake by these APC (Aucouturier et al., 2001; Aguilar and Rodriguez, 2007). There is a minute portion of bacterial proteins in the capsule, immunity against which can be enhanced by attaining required level in the dose and addition of adjuvants in the Bacterin. In this research study, 2mg bacterial dry mass per mL of the growth was achieved by using enrichments media and filtered air during incubating process. These results agree with the study of Afzal and Muneer (1990); Tariq et al., (1997).

In this research study, Brain Heart Infusion (BHI) broth was used for growth of P. multocida which is in accordance with Khan et al., (2013) that BHI is highly enriched media that supports the growth of P. multocida as compared to Nutrient broth.

In cryopreservation technology, low temperature is used for preservation of intact living cells. Cryoprotectants work by decreasing the quantity of ice formation at any given temperature; increase the concentration of solutes in the system and having low toxicity. Glycerol and dimethyl sulfoxide have such properties (Pegg, 2007). Diffusion and osmosis are the major processes having influence in the addition, removal, freezing and thawing processes for cryopreservation (Pegg, 2007).

Cryopreservation has also emerged as promising technique for fertility preservation and assisted reproduction techniques for production of animal breeds and genetically engineered animal species for research (Aljaser, 2022).

Dimethyl sulfoxide and glycerol are permeating cryoprotectant agents which are smaller sized (less than 100 daltons) and are amphiphilic in nature. So these
molecules can enter through the cell membrane easily, try to equilibrate within the cytoplasm and replace the intracellular water to avoid excessive dehydration. In this way they save the cell from intracellular ice formation and salt accumulation (Aljaser, 2022).

In cryobiology, dimethyl sulfoxide is considered as a most effective, fast penetrating and universal cryoprotective additive (CPA) (Hubalek, 2003). Red blood cells and spermatozoa were cryoprotected originally by using dimethyl sulfoxide (Lovelock, 1959). It was also used for cryopreservation of bacteria (Gibson et al. 1966). The concentration of dimethyl sulfoxide (MeSO) varies from 1 to 32 % but 10 % concentration is considered as most suitable (Aljaser, 2022). The toxicity of MeSO is less at 0-5 °C as compared to higher temperatures. Low temperature should be used while freezing the samples with MeSO. The most widely used CPA in microbiology is the combination of glycerol and MeSO (Hubalek, 2003). Thus selection of appropriate CPA concentration is vital for maintaining structural integrity and viability after freezing (Aljaser, 2022).

The results of cryopreservation of *P. multocida* pure growth with different cryo-preservatives at -20 °C after three months are tabulated in Table 1.

### Table 1: Results showing *P. multocida* survival with and without cryo-protectant at – 20 °C after 3 months

<table>
<thead>
<tr>
<th><em>P. multocida</em> + Cryo-protectants</th>
<th>Microscopic Examination</th>
<th>Purity Test</th>
<th>Optical Density Value (430 nm)</th>
<th>Dry Biomass Calculation</th>
<th>Mice inoculation Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part-A</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Live</td>
</tr>
<tr>
<td>Part-B</td>
<td>Positive</td>
<td>Positive</td>
<td>2.4</td>
<td>2.2 mg/mL</td>
<td>Died</td>
</tr>
<tr>
<td>Part-C</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Live</td>
<td></td>
</tr>
</tbody>
</table>

**Part-A:** Pure *P. multocida* + 5 % DMSO  
**Part-B:** Pure *P. multocida* + 10 % DMSO +15 % Glycerine  
**Part C:** Pure *P. multocida*

The results of this study indicated that pure *P. multocida* culture mixed with 10 % sterile DMSO and 15 % sterilized glycerol stored at – 20 °C survived for three months and can be used for HS vaccine production without losing its survival. Our results are in accordance with the findings of Moore et al. (1995), Hubalek (2003), (Aljaser, 2022) and Marquez-Curtis et al. (2022) who used DMSO (10 %) and glycerol as Cryoprotectants for preservation of *P. multocida*, sperm, ova and human islet cells.

Bicot et al. (2022) found that when compared to glycerol, which has been shown to form ice crystals inside cells potentially leading to cell lysis during thawing, DMSO generally allows better recovery of a wide range of microorganisms. Franco et al. (2021) recommend that using sufficiently large community aliquots for cryopreservation will improve the reproducibility and long-term storage times from 6 up to 12 months did not seem to cause major changes in the community composition of the resuscitated communities. The research findings of Nikitin et al. (2022) are in agreement with our findings who used 10 % glycerol for cryopreservation and found that glycerol provides 98.6 % survival of *M. haemolytica*.

### REFERENCES


Almoheer, R., M.E. Abd Wahid, H.A. Zakaria, M.A.B. Jonet, M.M. Al-shaibani, A.


