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

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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

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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 Sulf-Oxide (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 found 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 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) 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 (OBV) 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 Rodiguez, 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 (Me_2SO) varies from 1 to 32 % but 10 % concentration is considered as most suitable (Aljaser, 2022). The toxicity of Me_2SO is less at 0-5 °C as compared to higher temperatures. Low temperature should be used while freezing the samples with Me_2SO. The most widely used CPA in microbiology is the combination of glycerol and Me_2SO (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

***P.multocida* + Cryo-protectants**	**Microscopic Examination**	**Purity Test**	**Optical Density Value (430 ηm)**	**Dry Biomass calculation**	**Mice inoculation Test**
Part-A	Negative	Negative	Negative	Negative	Live
Part-B	Positive	Positive	2.4	2.2 mg/mL	Died
Part-C	Negative	Negative	Negative	Negative	Live

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.

Biclot *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

Abbas, A.K., A.H. Lichtman and J.S. Pober (1991). Cellular and molecular immunology, W.B. Saunders Company, London, UK. 417p.

Afzal, M. and R. Muneer (1990). Development of combined vaccine of haemorrhagic septicaemia and foot and mouth disease. Pak. Vet. J., 10: 67-69.

Aguilar, J.C. and E.G. Rodriguez (2007). Vaccine adjuvants revisited. Vaccine, 25: 3752-62.

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

Al-Gheethi, S.N.K. Addis (2022) Spatial, Temporal, and Demographic Patterns in the Prevalence of *Hemorrhagic Septicemia* in 41 Countries in 2005–2019: A Systematic Analysis with Special Focus on the Potential Development of a New-Generation Vaccine. Vaccines. 10(2): 315.

Aljaser,F.S. (2022) Cryopreservation methods and frontiers in the art of freezing life in animal models. Chapter in Book Animal Reproduction. DOI: 10.5772/intechopen.101750

Aucouturier, J., L. Dupuis and V. Ganne (2001). Adjuvants designed for veterinary and human vaccines. Vaccine, 19: 2666-2672.

Biclot, A., G.R.B. Huys, R. Bacigalupe, D. Kevin, D. Vandeputte, G. Falony, R.Y. Tito and J. Raes (2022). Efect of cryopreservation medium conditions on growth and isolation of gut anaerobes from human faecal samples. Microbion, 10:80.

Bain, R.V.S., M.C.L. De Alwis, G.R. Carter and B.K. Gupta (1982). Haemorrhagic Septicaemia. FAO Publication, Rome, Italy.

Bratbak, G. and I. Dundas (1984). Bacterial dry matter content and biomass estimations. Appl. Environ. Microb., 48 : 755-757.

Farooq, U., Z. Saeed, M.A. Khan, I. Ali and M.F. Qamar (2011). Sero-surveillance of Haemorrhagic

Septicaemia in buffaloes and cattle in Southern Punjab, Pakistan. Pak. Vet. J., 31: 254-256.

Franco, A.R., G.P. Moraes and S. Beier (2021). Cryopreservation and resuscitation of natural aquatic prokaryotic communities. Front. Microbiol. 11:597653

Gibson, C.A., G.B. Landerkin and P.M. Morse (1966). Effects of additives on the survival of lactic streptococci in frozen storage. Appl. Microb., 14: 665-669.

Hubalek, Z. (2003). Protectants used in the cryopreservation of microorganisms. Cryobiol., 46: 205-229.

Khan, A., M.K. Saleemi, M.Z. Khan, S.T. Gul, M. Irfan, and M.S. Qamar (2011). Haemorrhagic Septicaemia in buffalo (*Bubalus bubalis*) calves under sub-tropical conditions in Pakistan. Pak. J. Zool., 43: 295-302.

Khan, A., S. Munir, Q. Jamal, M. Anees, S.A. Shah, S.K. Sherwani, A. Basit, G. Ali, Asadullah and M. Hussain (2013). Biomass production of *Pasteurella multocida* using biofermenter. Int. J. Adv. Res., 1:142-151.

Lovelock, J.E. and M.W.H. Bishop (1959). Prevention of freezing damage to living cells by di-methyl-sulph-oxide. Nature, 183:1394-1395.

Marquez-Curtis, L.A., X. Q. Dai, Y. Hang, J.Y. Lam, J. Lyon, J.E.F. Manning, *et al*. (2022). Cryopreservation and post-thaw characterization of dissociated human islet cells. PLoS ONE 17(1): e0263005. https://doi.org/10.1371/ journal. pone.0263005

Moore, M.K., D.J. Shadduck, D. A. Goldberg and M. D. Samuel (1995). A Cryopreservation method for *Pasteurella multocida* from Wetland samples. J. Wildlife Dis., 34: 152-155.

Nikitin, G., S. Makavchik, A. Sukhinin and L. Smirnova (2022). Aspects of *Mannheimia haemolytica* cryopreservation. FASEB. 36: https://doi.org/10.1096/fasebj.2022.36.S1.R3107

O.I.E. (2018). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Office De International Epizootics, Paris, France. 732-745p.

Pegg, D.E. (2007). Principles of cryopreservation. Methods Mol. Biol., 368: 39-57.

Shah, A.H., A.A. Kamboh, N. Rajput and N.A. Korejo (2008). Optimization of Physico-chemical conditions for the growth of *Pasteurella multocida* under in-vitro. J. Agri. Soc. Sci., 4:176-79.

Shahzad, W., R. Munir, M. Asif, M. S. Sagar, M. Altaf, W. Aslam, G. Akbar and F. Mehmood (2013). Prevalence, molecular diagnosis and treatment of field isolates of toxogenic *Pasteurella multocida* in a Haemorrhagic Septicaemia outbreak in Nili-Ravi buffalo calves at Livestock Experiment Station, Bahadurnagar, Okara, Pakistan. *Buffalo Bull.*, 32:1046-1050.

Tariq, M.A., K. Muhammad, P. Das and M.A. Sheikh (1997). Preparation and evaluation of alum precipitated and oil-based Haemorrhagic Septicaemia vaccines. Pak. Vet. J., 17:175-78.

Thomson, C.M.A., N. Chanter and C.M. Wathes (1992). Survival of Toxigenic *Pasteurella multocida* in aerosols and aqueous Liquids. Applied Environ. Microb., 58: 932-936.

Watko, L.P. and K.L. Heddleston (1966). Survival of shell-frozen, freeze-dried and agar slant cultures of *Pasteurella multocida*. *Cryobiol*., 3: 53-55.

Xie, J., M.D. Ekpo, J. Xiao, H. Zhao, X. Bai, Y. Liang, G. Zhao, D. Liu and S. Tan (2022). Principles and Protocols for Post-Cryopreservation Quality Evaluation of Stem Cells in Novel Biomedicine. Front. Pharmacol. 13:907943.