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Original Article

Intradermal Inoculation with Formalin Treated Whole Cell Proteus mirabilis Boosts up Protective Immunity by Enhancing Memory B Cell Response in Swiss Albino Mice

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Abstract

Background: *Proteus mirabilis is an important opportunistic human pathogen capable of causing a wide range of infections. With MDR P. mirabilis infections on the rise, especially on those underwent long-term indwelling urinary catheterization, the need for a rationally designed vaccine against this pathogen is critical. This study was conducted to evaluate the protective efficacy of antibodies elicited by formalin inactivated vaccine against MDR P. mirabilis and to observe the memory B cell response for future protection.*

Materials and methods: *In this experimental study, MDR P. mirabilis isolated from different clinical samples were used in preparing formalin inactivated whole-cell P. mirabilis and employed Intradermally (I/D) in experimental group (Group-1) mice thrice at 14 days interval. Two weeks after 3rd dose of immunization, group-1 and group-2 mice were intraperitoneally challenged with live P. mirabilis and observed for 14 days. Tail blood was collected 7 days after each booster and followed by cardiac puncture 14 days post challenge. Antigen binding capacity of the protective IgG was determined by ELISA and memory B cell evaluation was done on RPMI media.*

Results: *All immunized mice in group-1 (100%)survived after the lethal dose of live P. mirabilis. When antigen binding capacity was evaluated by ELISA, all the pre-challenge and post challenge immunized serum IgG antibody of experimental group mice showed significantly higher Optical Density (OD) values compared to the OD values of control mice sera.Blood was collected from group-1 and negative control (Group-3) by cardiac puncture14 days post challenge. Peripheral Blood Mononuclear Cells (PBMCs) were separated from the blood by using density gradient centrifugation on Ficoll-isopaque, and incubation of the cells were done in RPMI media. Cell culture supernatant samples were used from each group to measure IgG antibody absorbance by ELISA to evaluate memory B cell response.*

Conclusion: *Statistically significant difference between the OD values of experimental and control mice cell culture supernatant was observed which is suggestive of significant production of memory B cells that might provide long term immunity for combating MDR P. mirabilis infections.*

Key words: ELISA; IgG; Intradermal; Immunization; Memory B cell; OD value; PBMCs.

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INTRODUCTION

Proteus mirabilis, a Gram negative highly motile bacteria is found to be an emerging threat to both animals and humans for a variety of infections.[1] The difficulty in treating P. mirabilis and the increase in antibiotic resistant infection emphasize on developing P. mirabilis vaccine. The vaccine would be beneficial for population

more prone to P. mirabilis UTI, including individuals with functional or structural urinary tract abnormalities or individuals starting long time catheterization.[2] In order to improve the presentation of multiple antigens to the immune system, we might focus on development of wholecell killed or live-attenuated vaccines. For an attempt to prevent drug resistant infections, deactivation method could be simultaneously applied as an innovative strategy for development of bacterial vaccine and for which formaldehyde is an effective deactivating agent.[3]

The dermal layers are easy access biological sites with great immunological relevance for the abundant presence of Langerhans Cells (LCs) Dendritic Cells (DCs) and other Antigen Presenting Cells (APCs). Theepidermis is populated byLCs which are specialized APCs characterized by the expression of langerin. Langerin is also expressed in subpopulations of DCs and migrating LCs in the dermis and within the skin draining lymph nodes. So, as per immunological point of view, skin is a very attractive site for vaccine delivery. Most antigens delivered to the skin are captured by APCs which migrate to skin draining lymph nodes. Several subsets of dermal DCs are observed in both human and mouse dermis.[4]

One of the hallmarks of our immune system is the ability to remember past exposure to the pathogens and memory B and T cells are critical in secondary responses to infections.[5] The role of B cells in generating effective immune response to vaccine is unquestioned; the most effective vaccines generate protective long-lived humoral immune response.[6]It is generally accepted that this long-term humoral immunity is a product of both long-lived plasma cells that secrete antibodies and Memory B-Cells (MBCs). MBCs are defined as long-lived and quiescent cells that are poised to quickly respond to antigen upon recall.[5] They rapidly and specifically respond to antigenic stimulation, thus contributing to both the short-lived and long-lived plasma cell pool and thereby prolonging the period of high serum antibody levels.[6]

However, no study regarding MBC response in mice after vaccination againstP. mirabilishas been carried out in Bangladesh so far. So, this study has been designed to evaluate the MBC response after I/D immunization with formalin inactivated whole cellP. mirabilis in murine infection model.

MATERIALS AND METHODS

This experiental study was conducted at the Department of Microbiology, Dhaka Medical College (DMC) Dhaka during the period from January to December 2022.

Animals

Fifteen 4-6 weeks old female Swiss albino mice were collected from Animal Resources Facility of ICDDRB and were kept and cared in animal house facility of Microbiology Department of the DMC. The mice were randomly divided into 3 groups having 5 mice in each; experimental (Group-1) placebo control (Group-2) and negative control (Group-3) and were inoculated with formalin inactivated whole cell Proteus mirabilis, PBS (Phosphate Buffer Saline) respectively and group-3 was kept uninoculated and uninfected.Non-medicated feed and water were provided throughout the experiment. An animal study was approved by the ethical review committee of the respective institution.

Immunization of mice

Bacterial Culture

All bacterial cultures were maintained at 37°C for 24 hours before each useto ensure that the bacteria wereat the same growth stage in all experimental steps.

Preparation of formalin inactivated whole-cell Proteus mirabilis

Previously isolated P. mirabilis strains were collected from different clinicalsamples and used as candidate for inactivated whole-cell vaccinepreparation.A loop full of P. mirabilis was inoculated into TSB (Trypticase soy broth) in micro centrifuge tube and was incubated at 37°C overnight. Following incubation, the bacterial cultures were centrifuged and the supernatant was discarded. The pelleted bacteria were then washedwith ice cold PBS. To prepare 1 ml of formalin inactivated whole-cellP. mirabilis, 70µl of 37%formalin was added to the suspension to achieve final concentration of 3% (v/v) (By using the formula $m_1v_1 = m_2v_2$). The suspension was incubated for 2 hours, again washed and resuspended with ice-cold sterile PBS to achieve conc. of 1.5×10^8 CFU/ml which was determined by comparing with 0.5 McFarland turbidity standard. By using the formula final concentration is achieved. Complete inactivation of the bacteria was checked by streaking on the Muller Hinton agar plates and confirmed by observing no growth after overnight incubation and stored at -20°C until inoculation.

Immunization Schedule

Three intra dermal (20µl I/D) inoculations were performed using an insulin syringe BD Ultra-Fine TM (31G) on day 0, 14 und 28 in the alternate thigh of the group-1 mice with the prepared suspension and the group-2 mice with sterile PBS. The vaccination was done after giving proper anesthesia with intra-peritoneal injection of ketamine adjusted to the body weight of mice (100 mg/kg).

Collection of Serum for ELISA

Serum from tail blood was collected 10 days after 1st inoculation and 7 days after each inoculation to detect OD value of IgG antibody absorbance by ELISA. Ketamine was used as anesthetic agent. Then the tail was cleaned with 70% alcohol and cut 2 mm proximal to its blunt end with a sterile scalpel (22 FR). 10 µloffresh blood was collected into anEppendorf tube containing 40 µl PBS to yield adilution of 1:5. The diluted sera were kept upright for 2 hours followed by

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centrifugation at 3,000 g for 10 minutes. Blood cell settled down at the bottom and clear sera from thetop of the tube was taken into a separate Eppendorf tube and was kept at -20°C for further use.

Intra-peritoneal Challenge

Two weeks after the last inoculation, the mice from group 1 and 2 were challenged intraperitoneally with1.5X10^8 CFU/ml liveP. mirabilisin 100µl PBS. All mice were observed for 14 days post challenge for any clinical manifestations such as weight loss, lack of movement, reluctant to feed or death.

Collection of Blood after Cardiac Puncture

Fourteen days after the lethal challenge, blood was collected by cardiac puncture from the mice of group-1 and group-3. The chest area was shavedfollowed by washing with povidone iodine and 70% alcohol. Cardiac pulsation was felt by finger and about 3 ml blood was drawn from heart by insulin syringe introduced at an angle of 45° after proper anaesthetization. About 1ml of blood was collected keeping undiluted in a sterile test tube and allowed to clot itself. Centrifugation was done at 3000 g for 10 minutes and serum was collected from the top in a micro centrifuge tube and kept at -20°Ctemperature for further use. Rest of the blood was collected in heparin tube for further separating the PBMC.

Separation of Peripheral blood mononuclear cell[7,8]

The blood that was taken in a heparinized blood collection tubewas diluted with PBS in 1:1 ratio (Blood: PBS=1:1). Then the diluted blood was poured slowly in each of the falcon tubes where already 1ml ficoll-isopaque has been taken and centrifugation was done at 4000g for 30 minutes. A clear layer of PBMCwas seen above the ficoll-isopaque which was taken with the help of micropipette.

Viability of cell[9]

Centrifugation of an aliquot of cell suspension being tested for viability, was done for 5 min at 100g and supernatant was discarded.The cell pellet was resuspended in PBS.One part of 0.4% trypan blue was mixed withone part of cell suspension andincubatedfor 3 mins at room temperature and applied to hemocytometer for observation under microscope. The unstained (Viable) and stained (Nonviable) cells were separately counted in the hemocytometer using the following formula:

Viable cells % = (Total number of viable cells per ml of aliquot /total number of cells per ml of aliquot) × 100

The samples were considered appropriate if >50% cells were found viable.

Harvesting PBMC in RPMI media[10]

Clear layer of PBMC was taken with micro pipette and added in a 24 well culture plate which contained 1 ml of RPMI media and 50µl of diluted Ag in each well. The culture medium contained RPMI 1640 medium (Only part-A) 10% fetal bovine serum, 200U/ml Penicillinand 200µgm/ml Gentamicin. Incubation of the plate was done at 37°c for 6 days.

Detection of IgG Ab in mice serum and from cell culture supernatant of RPMI mediaby ELISA

Mice sera and freshly harvested PBMCs from RPMI media were used for evaluation of OD value regarding the titer ofIgG by ELISA. Antigen were separated after sonicating the *P. mirabilis*. Absorbance was measured at 450nm using ELISA plate reader (Biotek Inc., USA).10 µg/ml of Ag wasconsidered as ideal quality to use in indirect ELISA study.

Measurement of Optical Density (OD)

Cut off value of OD was calculated using given below formula:

OD= M (mean) + 2 × SD (Standard deviation).

All data were compiled and edited meticulously by thorough checking and rechecking. Statistical significance of serumAb titer and MBC response between experimentaland control group was determined by Student's t-test. Statistical significance of serum Ab titer within different inoculation schedule of experimental group was determined by Anova test. Allstatistical analyses were done by Microsoft Excel (2019). P value <0.05 was taken as minimal level of significance.

RESULTS

Table I Survival of mice after 14 days of post challenge

Survived/Dead	Group-1 (n=5)	Group-2 (n=5)
Survived	5 (100%)	0 (0.00%)
Dead	0 (0.00%)	5 (100%)

Group-1 =Immunized/Infected.

Group-2= Unimmunized/Infected.

The survival rate of immunized and unimmunized mice after lethal challenge. All the mice of group-1 survived during 14 days observation period after challenge. All the mice of group-2 died within 24 hours of challenge (Table I).

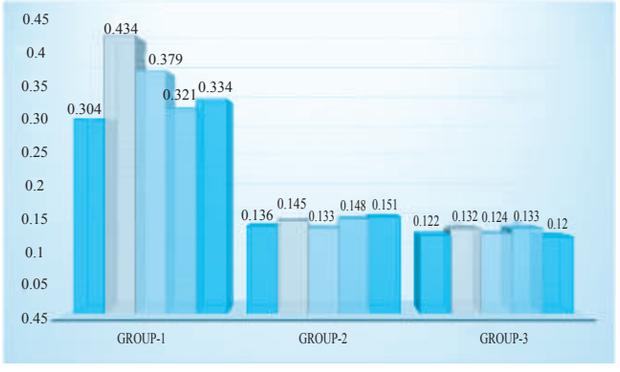

Figure 1 Optical Density (OD) of serum samples after 1st booster by ELISA

Here, mean of the negative control 0.1262, Standard Deviation (SD)0.006, Cut off value for pre-challenge sera 0.1382, Range after first booster 0.304-0.434, p value < 0.001

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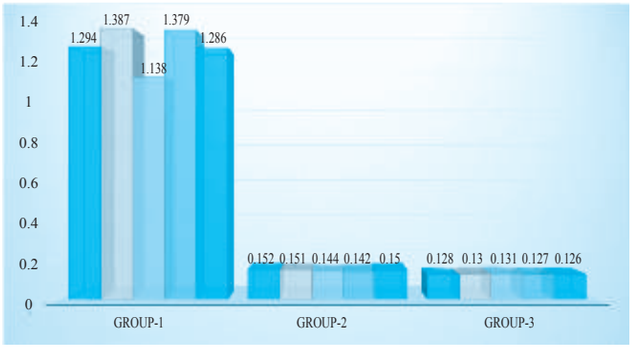

Figure 2 Optical Density (OD) of serum samples after 2nd booster by ELISA

Here, mean of the negative control 0.1284, SD value 0.002,Cut off value for pre-challenge sera 0.1324, Range after second booster 1.138-1.387, p value < 0.0001.

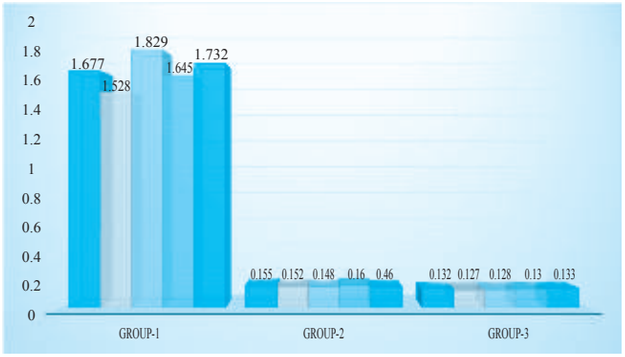

Figure 3 Optical Density (OD) of serum samples after 3rd booster by ELISA

Here mean of the negative control 0.13, SD value 0.003, Cut off value for pre-challenge 0.136
Range after 3rd booster 1.528-1.829, p value < 0.0001.

Figure 4 Optical Density (OD) of serum samples after lethal challenge by ELISA

Here, mean of the negative control 0.1278, SD value 0.006, Cut off value for post challenge 0.1398, Range after lethal challenge 1.365-1.524, p value < 0.00001, All the mice of Group-2 died within 24 hours after lethal challenge.

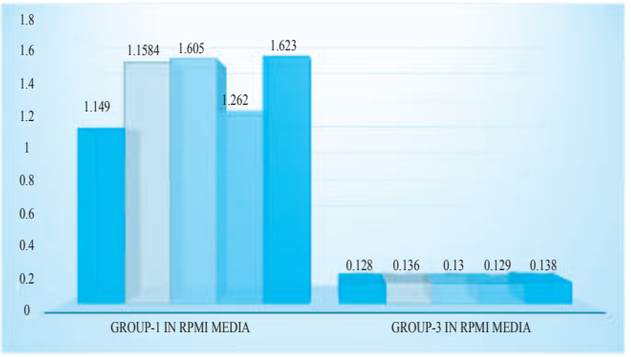

Figure 5 Optical Density (OD) of cell culture supernatant after harvesting PBMC in RPMI media, by ELISA

Here, mean of the negative control 0.1322, SD value 0.004, Cut off value for negative control 0.1402, Range1.149-1.623, P value is .000179 which is less than 0.05. So, there is significant difference between OD values of cell culture supernatant of experimental group and negative control group. In other words, there is significant production of memory B cells within the experimental group as compared to the control group.

DISCUSSION

Very limited studies have been documented on Proteus mediated infections. In recent years, researchers are involving in studies regardingProteus spp. because of it's expanding profile on antibiotic resistance. Worldwide, *P. mirabilis* has developed resistance to several classes of antibiotics complicating treatment. So, it is becoming very essential to develop an effective vaccine against *P. mirabilis* that could prevent infections caused by highly antibiotic-resistant microorganism.[2]

In this study, formalin inactivated *P. mirabilis* was used to immunize the experimental group mice and survival proportion among the experimental mice was 100% at 14 days post challenge. A study reported 100% survival of mice after giving formalin inactivated whole cell *P. mirabilis* vaccination intra nasally.[11] In present study, the mice from experimental group survived while all the mice from the control groupdied after lethal challenge as because the serum IgG was insufficient for them to survive. No data are available regarding survival rates of mouse after lethal challenge following intradermal immunization with formalin inactivated *P. mirabilis.* In this study, formalin inactivated whole cell bacteria were used in order to improve the presentation of multiple antigens to the immune system and stimulate broader immune response. One study showed immunization with formalin inactivated whole cell vaccine elicited significant levels of IgG in immunized mice group than the control group.[12] Another studyreported that formalin inactivated bacteria induced IgGproduction after vaccination.[3]

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Intradermal route was selected because skin is the largest immunological organ in the body which harbor large number of T lymphocytes and densely populated by APCs.[4] A study in Bangladesh suggested I/D immunization to be one of the promising routes of immunization that produce large number of protective Ab in experimental BALB/c mice.[13] Another study in Bangladesh observed production of protective antibody in experimental group mice after oral administration of formalin inactivated whole cell vaccine but to a lesser extent than I/D route.[14]

In current study, after each inoculation sera were collected for analyzing IgG Ab titer. There was gradual increase in Ab titer after each inoculation in the experimental group mice which is similar to finding of one of the studies in Bangladesh.[13] However, the Ab level slightly decreased following lethal challenge which might be due to utilization of antibodies to clean up offending pathogens from the body. The sera from group-2 mice contained very low level of detectable Ab after all inoculation which was close to negative control (Group-3) mice. Two other studies in Bangladesh also observed similar trend in their studies.[13,14]

These antibodies in the serum can be crucial to the activation of the classical pathway of the complement system in the early stages of infection, thus the efficient depuration of the bacteria.[13] Current study showed that, after the second booster the OD values of the IgG Ab were highest for pre-challenge mouse sera. This could be due to the fact that after the second booster, more IgG antibodies were produced by the memory cells. After the first booster and following lethal challenge, the values were less as antibodies were used up for clearing the harmful pathogen from the body.

Followingcardiac puncture, blood was collected from group-1 and 3. ThePBMCswere then separated from the blood using density gradient centrifugation on Ficoll-Isopaque. In a study in Bangladesh, they also used the Ficoll-Isopaque for centrifugation and separating PBMC from heparinized blood after cardiac puncture.[15] In this study, incubation of the PBMC was done in RPMI media and the IgG titer was measured by ELISA which showed high IgG titer in experimental group mice which is an indirect evident to the production and differentiation of MBCs following immunization. Another study in Bangladesh also showed ≥4-fold increase in the number of total IgG memory cells following culture of the PBMC in RPMI media.[15]

CONCLUSION

In current study, there is increase in IgG titer after vaccination in mice serum and also in the PBMC culture supernatant. Since B cells are responsible for producing IgG, it can be considered that there has been increased proliferation and differentiation of memory B cells due to antibody mediated response following immunization causing rise in IgG titer.

DISCLOSURE

All the authors declared no competing interest.

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REFERENCES

1. Lv P, Hao G, Cao Y, Cui L, Wang G, Sun S. Detection of carbapenem resistance of proteus mirabilis strains isolated from foxes, raccoons and minks in China. Biology. 2022;11(2):292.
2. Schaffer JN, Pearson MM. Proteus mirabilis and urinary tract infections. Urinary tract infections: Molecular pathogenesis and clinical management. 2017;383-433.
3. Fan Y, Mu Y, Lu L, Tian Y, Yuan F, Zhou B. Hydrogen peroxide-inactivated bacteria induces potent humoral and cellular immune responses and releases nucleic acids. International Immunopharmacology. 2019; 69:389-397.
4. Pulit-Penaloza JA, Esser ES, Vassilieva EV, Lee JW, Taherbhai MT, Pollack BP, Prausnitz MR, Compans RW, Skountzou I. A protective role of murine langerin+ cells in immune responses to cutaneous vaccination with microneedle patches. Scientific reports. 2014;4(1):6094.
5. Palm AK, Henry C. Remembrance of things past: long-term B cell memory after infection and vaccination. Frontiers in immunology. 2019; 10:1787.
6. Sebina I, Cliff JM, Smith SG, Nogaro S, Webb EL, Riley EM, Dockrell HM, Elliott AM, Hafalla JC, Cose S. Long-lived memory B-cell responses following BCG vaccination. PloS one. 2012;7(12): e51381.
7. Puleo A, Carroll C, Maecker HT, Gupta R. Isolation of Peripheral Blood Mononuclear Cells Using Vacutainer ((R)) Cellular Preparation Tubes (CPT (TM)). Bio Protoc. 2017; 7: e2103 [Internet].
8. Seeligmüller N. Faster Isolation of PBMC Using Ficoll-Paque® Plus in the Eppendorf Multipurpose Benchtop Centrifuges 5920 R and 5910 Ri. Eppendorf Application Note. 2021.
9. Strober W. Trypan blue exclusion test of cell viability. Current protocols in immunology. 2015;111(1): A3-B.
10. Leung DT, Rahman MA, Mohasin M, Patel SM, Akter A, Khanam F et al. Memory B Cell and Other Immune Responses in Children Receiving Two Doses of an Oral Killed Cholera Vaccine Compared to Responses following Natural Cholera Infection in Bangladesh. Clinical and Vaccine Immunology, 2012; 19 (5): 690-698.
11. Li X, Lockatell CV, Johnson DE, Lane MC, Warren JW, Mobley HL. Development of an intranasal vaccine to prevent urinary tract infection by Proteus mirabilis. Infection and immunity. 2004;72(1):66-75.
12. Takahashi K, Hanamura Y, Tokunoh N, Kassai K, Matsunishi M, Watanabe S, Sugiyama T, Inoue N. Protective effects of oral immunization with formalin-inactivated whole-cell Citrobacter rodentium on Citrobacter rodentium infection in mice. Journal of Microbiological Methods. 2019; 159:62-68.
13. Kawser Z, Shamsuzzaman SM. Intradermal immunization with heat-killed Klebsiella pneumoniae leading to the production of protective immunoglobulin G in BALB/c mice. International Journal of Applied and Basic Medical Research. 2021;11(3):160-165.
14. Rahman A, Shamsuzzaman SM, Dola NZ, Nabonee MA. Protective Effects of Immunization with Formalin Inactivated Oral Whole Cell Vaccine against Multidrug Resistant Citrobacter freundii in Murine Model. American Journal of Infectious Diseases and Microbiology. 2022; 10:92-97.
15. Harris AM, Bhuiyan MS, Chowdhury F, Khan AI, Hossain A, Kendall EA, Rahman A, LaRocque RC, Wrammert J, Ryan ET, Qadri F. Antigen-specific memory B-cell responses to Vibrio cholerae O1 infection in Bangladesh. Infection and immunity. 2009;77(9):3850-3856.

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