Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Rabies Prophylaxis Strategy in Iran, a Need for an Alternative Strategy: Would the Essen Regimen be Replaced by Zagreb Protocol?

Pooneh Rahimi [1], Rouhollah Vahabpour [2], Mohammad R. Shirzadi [3], Golnaz Bahramali [1], Mohammad R. Aghasadeghi [1]

[1] *Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran*
[2] *Medical Laboratory Technology Department, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3] *Center of Disease Control (CDC), Ministry of Health, Tehran, Iran*

SUMMARY

Background: **Rabies is a fatal zoonotic infectious disease, which can be prevented by prompt post-exposure prophylaxis that could be expensive in countries with a large population. The Essen protocol with injection of 5 single doses of human rabies vaccine on separate days is a well-known rabies prophylaxis schedule. Decreasing the number of vaccine doses and the number of clinical visits due to an effective alternative schedule is strongly needed. The 2-1-1 regimen, known as Zagreb, is one of the best candidates to succeed Essen.**
Methods: **To evaluate the effectiveness of Zagreb regimen in the Iranian population by using the Purified Vero cell Rabies Vaccine (PVRV), anti-rabies antibody titer was measured in volunteers with second and third exposure through Rapid Fluorescent Focus Inhibition Test (RFFIT) and Enzyme Linked Immunosorbent Assay (ELISA) test and compared with patients, who were treated according to the Essen protocol.**
Results: **In all participants, anti-rabies antibody titer reached the protective level with no suppressive effect of rabies immunoglobulin in patients with third exposure in Zagreb regimen.**
Conclusions: **Zagreb regimen could be considered a suitable alternative for the Essen protocol.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2018.180619)

Correspondence:
Pooneh Rahimi
Department of Hepatitis and AIDS
Pasteur Institute of Iran
Farvardin 12
Enghelab square
Tehran
Iran
Phone: +98 21-66969291
Fax: +98 21-66969291
Email: prahimi@pasteur.ac.ir
ORCID ID: 0000-0001-8829-5373

Manuscript accepted August 2, 2018

KEY WORDS

rabies post-exposure prophylaxis, 2-1-1 regimen, Zagreb protocol, Essen protocol

INTRODUCTION

Rabies is an acute and progressive encephalomyelitis, which is the most important deadly zoonotic disease caused by an RNA virus belonging to the Rhabdoviridae family and genus Lyssavirus. The virus sheds in saliva of an infected animal at a high concentration and is transmitted through direct contact by the bite or scratch of a rabid animal to other animals or humans [1-4]. In order to better understand the risks of infection and performing appropriate anti-rabies prophylaxis strategy, the World Health Organization (WHO) categorized types of rabies exposure as: category I - touching or feeding animals, licks on the skin; category II - nibbling of uncov-

ered skin, minor scratches or abrasions without bleeding, licks on broken skin; and category III - single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks; exposure to bat bites or scratches [2,3]. Despite its deadly nature, it could be prevented by avoiding viral exposure and administration of proper prophylaxis strategy using potent rabies vaccines, according to the World Health Organization recommendations [1,4-6]. There are two main post-exposure prophylaxis protocols with Intramuscular (IM) administration of Purified Vero-cell Rabies Vaccines (PVRV) [1-3]. The traditional Essen protocol consists of five single doses of human rabies vaccine injected intramuscularly over a 28-day period, which has been modified in order to decrease the number of injections to four doses and also, the number of clinic visits. It was found effective, especially in immunocompetent individuals, thus it must be considered as an alternative Rabies Post-Exposure Prophylaxis (RPEP) protocol with precaution [5,6]. The Zagreb regimen, also known as the 2-1-1 protocol, consists of bilateral administration of vaccine on day zero, followed by a single dose on day seven and 21 [5-7]. In both protocols, when a category III exposure takes place, injection of anti-rabies immunoglobulin on day zero is mandatory, as strongly recommended by WHO [2,3,5,6]. Although human rabies has dropped in Iran during the past few years due to a well-organized surveillance system, continuing health education, and the availability of a potent human rabies vaccine, there is still a strong need for an alternative rabies vaccination schedule, which results in a decrease in the cost of RPEP. This project was conducted following a previous study, which was done as a pilot study on 25 volunteers with category II exposure [11]. Herein, the current research evaluated the effectiveness of the Zagreb regimen in comparison with the traditional Essen regimen in RPEP in volunteers with second and third category type of exposure (according to the WHO categories of rabies exposures), who had been bitten by their pets.

MATERIALS AND METHODS

There were 42 and 30 healthy volunteers with 18 - 60 years old who experienced animal bites in second and third categories of rabies exposure according to the WHO recommendations, respectively [1,12]. The clear and complete information such as the RPEP with focus on especially Zagreb, the aims of this study, and the number of required blood specimens was given to both groups of volunteers. Those patients with exposure of 3rd category had been bitten by their pet animal. We named these 2 groups of volunteers as Zagreb II and Zagreb III according to the WHO's category of rabies exposure [1,12]. Also, we collaborated with another 15 healthy volunteers with 3rd rabies exposure experience who were under treatment with the Essen regimen. All patients were under treatment in the Department of Vaccine, Rabies Treatment and Prophylaxis, Pasteur Institute of Iran. The rabies vaccine injected to all participants was PVRV and the immunoglobulin used for treatment in 3rd exposure was Human rabies immunoglobulin (HRIG), and the dosages were in accordance with WHO recommendations [1,12]. There were inclusion and exclusion criteria for selecting the volunteers: they had no history of immunodeficiency or hypersensitivity to any vaccine and were immune-competent, all of them had no history of rabies vaccination, were not taking immune-suppressive or Choloroquine, had not been immunized with other killed or live vaccines within the last 3 weeks of rabies vaccination, and did not have any acute febrile disease. For female volunteers, pregnancy and breast feeding were considered exclusion criteria. All volunteers were injected intramuscularly with 0.1 mL of PVRV as it has been used in PREP strategy for many years in our country. This project was designed according to the Helsinki Declaration, the aim of the project and the blood sampling procedures were explained to the patients clearly and the consent form was signed by each volunteer or their official custodian. This study was approved by the Ethical Committees of Pasteur Institute of Iran, and the committee of Rabies Control, Ministry of Health, Treatment and Medical Education of Iran.

During this study, three blood samples were taken from all volunteers in each group on days 0, 21, and 35 (14 and 7 days after the last anti-rabies vaccination, respectively).

The rapid fluorescent focus inhibition test (RFFIT) was used for measuring the rabies neutralizing antibody titer as recommended and described by WHO using international reference sera as control [12,13]. Briefly, The BSR cells (a clone of baby hamster ovary cells) were grown and used in the test as described previously [11-16]. Sera were diluted at 1:3 in the 96-wells micro plates. A positive serum control standard diluted to a potency of 0.5 IU/mL and a negative serum control standard with a potency of < 0.1 IU/mL were prepared and included in each test. The international reference anti-rabies immunoglobulin with a known potency of 30 IU was diluted to a potency of 2.0 IU/mL to be used for comparison and expressing the results of anti-rabies antibody titers in IU/mL.

Diluted sera were mixed with a constant dose of challenge virus sufficient to cause infection in 80% of the cells and the mixture was incubated at 37°C for 1 hour. After incubation, susceptible cells were added to the serum-virus mixtures and incubated again for 24 hours. Then, the cellular monolayer was acetone fixed and stained with anti-nucleocapsid conjugate (Bio-Rad, France) according to the manufacturer's instructions to detect the presence of non-neutralized rabies virus (fluorescent foci). The fluorescent foci were counted and the results were calculated by using the Reed and Muench method [12,13]. The results of antibody titers were expressed in IU/mL in comparison with the international reference anti-rabies immunoglobulin serum

with a known potency of 30 IU that had been diluted to a potency of 2.0 IU/mL. A titer of > 0.5 IU/mL antibody was considered "protective" against rabies.
Herein, we also used an ELISA kit (Bio-Rad, Platelia, Rabies II, USA) which is based on the extracted rabies virus glycoprotein and a peroxidase conjugate (protein A from staphylococcus aureus) as an alternative method to determine anti-rabies neutralizing antibody titers in EU/mL according to the manufacturer`s instructions.

Statistical analysis
Data from both laboratory methods used in measuring anti-rabies antibody titer was analyzed using the repeated measure analysis of variance. Gender and age were considered as the covariates in this study; p-values < 0.05 were considered significant.

RESULTS

In two Zagreb groups males made up 31 (77.5%) and females made up 11 (27.5%) of 42 patients with second exposure and 22 (73.3%) males and 8 (26.7%) females (a total of 30 patients) were the volunteers with 3rd exposure. In the Essen group with 15 volunteers there were 9 (60%) males and 6 (40%) females. No serious adverse reactions were observed in this study. No participant had any detectable anti-rabies antibody titer prior to vaccination.
The geometric mean titer (GMT) was calculated for each group according to the type of exposure, RPEP (Zagreb or Essen), and the laboratory method used for anti-rabies antibody measurement on both blood samples collected on days 21 and 35, separately. The results are depicted in Table 1.
Successful seroconversion was confirmed in all participants in both Zagreb II and III and also in Essen group. Also, it remained at the protective level 1 week after the last vaccination. GMT results were also compared between males and females and between age groups and no statistically significant difference was observed (p > 0.05).

DISCUSSION

There are a few fatal diseases with no cure after initiation of symptoms and rabies, with approximately 60,000 deaths each year worldwide, is the most famous amongst them. However, it is almost completely preventable by using pre- and post-exposure prophylaxis and human rabies vaccines, such as PVRV, under different vaccination schedules, according to the WHO recommendations [5]. In Iran, the Essen protocol has been performed for many years with significant results in reducing human deaths. Yet still some RPEP failures occur that are the result of delay in clinic visits or even incomplete vaccination, especially when the distance between the patients' residences and the rabies treatment and prophylaxis centers is too far [14].
To reduce the cost of RPEP, many investigations have been performed to estimate the possibility of reduction in vaccine injections and number of clinic visits [5-10, 13,16-20].
Since rabies is an acute fatal disease, introducing a new RPEP schedule or replacement of the current RPEP strategy needs several extensive studies. Herein, this study was conducted on the effectiveness of Zagreb regimen in an Iranian population, and included both second and third category of exposures, because there were some reports about inappropriate effectiveness of Zagreb schedule in patients with third category exposure when injection with anti-rabies antibody is required, such as Human Rabies Immune Globulin (HRIG) [21-24].
To confirm that all participants in this study had no history of pre- or post- prophylaxis treatment, anti-rabies antibody titration was done on all blood samples collected in their first visit, prior to initiation of treatment. According to the WHO instructions for RPEP, the neutralizing antibody titer of ≥ 0.5 IU/mL, measured by RFFIT method, was considered as the anti-rabies protective antibody titer [12,24].
Herein, the results of the RFFIT method showed that all participants in this study in both Zagreb (II and III), and Essen groups reached the protective neutralizing antibody titer (RFFIT ≥ 0.5 IU/mL) and almost four times higher than that by day 21 (GMT = 17.83 IU/mL and 16.04 IU/mL in Zagreb II and III, respectively). Similar results were also observed by using an ELISA kit (2.87 EU/mL in Zagreb II and 2.1 EU/mL in Zagreb III). These results in Zagreb II patients were in accordance with many studies evaluating the effectiveness of the Zagreb regimen in different countries. However, the type of human rabies vaccine or HRIG used in some of the previous studies was different from those that were used for RPEP in Iran [6,8,18-24]. Also, the current results in Zagreb III participants was in contrast with a few reports about the suppression effect of rabies immunoglobulin injection on immune induction due to human rabies vaccine in patients with third category exposure, who received RPEP according to the Zagreb schedule [22-24]. On day 35, one week after the last vaccination, the anti-rabies antibody in all individuals who participated in this study still remained above the protective level, as identified by using RFFIT and ELISA. These observations were also in agreement with some other investigations [17,20-22]. In Zagreb II and III, according to the RFFIT test, the GMT was calculated as 23.54 IU/mL and 21.46 IU/mL, respectively, while it was 31.32 EU/mL in the Essen group. Also, in the Enzyme Linked Immunosorbent Assay (ELISA) test on day 35, successful seroconversion with anti-rabies antibody titer > 4 EU/mL was identified in all participants in both the Zagreb and Essen group. The Essen regimen is the most effective and safe RPEP, as proven by several investigations [2,3,6,8,11,16,17].
In the Zagreb group, GMTs showed slightly lower anti-

Table 1. Geometric mean titer of RPEP in patients with 2nd or 3rd category exposure under Zagreb or Essen schedule.

Type of RPEP [1]	Zagreb II [2]		Zagreb III [3]		Essen	
Day of blood sample collection→ Laboratory Method↓	Day 21	Day35	Day21	Day 35	Day 21	Day 35
RFFIT [4] (IU/mL)	17.83 (5.7 - 29.6)	23.54 (6.84 - 31.2)	16.04 (4.2 - 27.4)	21.46 (7.45 - 29.6)	28.41 (8.1 - 36.5)	31.32 (8.9 - 41.6)
ELISA (EU/mL)	2.87 (1.32 - > 4)	> 4	2.1 (0.83 - > 4)	> 4	3.1 (2.8 - > 4)	> 4

Two blood samples were collected on days 21 and 35 from volunteers with second category (nibbling of uncovered skin, minor scratches or abrasions without bleeding, licks on broken skin), and third category exposure (single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks; exposure to bat bites or scratches according to the WHO categories for rabies exposure), who were treated under Zagreb (2-1-1) regimen with injection of two doses of Purified Vero cell Rabies Vaccine (PVRV) on day zero and the remaining two doses were injected separately on days seven and 21. In the group of volunteers with third category exposure, anti-rabies immunoglobulin was injected on day zero. There was also a group of a few volunteers with third category exposure, who were treated under the Essen protocol with injection of single dose of rabies vaccine (PVRV) on days zero, three, seven, 14, and 28 and anti-rabies immunoglobulin on day zero. Geometric mean titer (GMT) was calculated according to the anti-rabies antibody titer measured using Rapid Fluorescent Focus Inhibition Test (RFFIT), which is presented in IU/mL and ELISA kit (Bio-Rad, Platelia, Rabies II, USA), which is presented in EU/mL.

[1] Rabies Post-exposure Prophylaxis.

[2] Group of volunteers with the 2nd category of rabies exposure, who received RPEP under the Zagreb regimen (2-1-1 vaccination protocol).

[3] Group of volunteers with the third category of rabies exposure, who received RPEP under the Zagreb regimen (2-1-1 vaccination protocol).

[4] Rapid Fluorescent Focus Inhibition Test.

body titer in individuals with third category exposure compared to those of second category exposure. However, there was no statistically significant difference in anti-rabies neutralization antibody titers, measured on day 21 (17.83 IU/mL versus 16.04 IU/mL) for second and third category exposure, respectively, and on day 35 (23.54 versus 21.46 IU/mL) for second and third category exposure, respectively ($p > 0.05$). These results were not in agreement with some reports of inhibitory effects of anti-rabies antibody injection on anti-rabies antibody production in patients with third category exposure, who were undergoing RPEP based on the Zagreb regimen [22-24].

Making a significant comparison of RPEP effectiveness between Zagreb and Essen regimens, this study included 15 volunteers with third category exposure. However, the results showed that the anti-rabies antibody titer was higher in these patients on both days 21 and 35 in comparison with the results of both Zagreb groups. These differences were not statistically significant ($p > 0.05$).

CONCLUSION

The Essen schedule is one of the most effective protocols and WHO approved the RPEP protocol, and it has been used for several years in Iran with successful reduction in the number of human rabies deaths. Despite all these advantages, searching for an effective and alternative RPEP with lower cost and number of clinic visits is important for developing countries. According to the results of this study, the Zagreb regimen elicited high titers of anti-rabies antibody in both second and third category exposure in the Iranian population with no adverse effects in the third category exposure; thus, it could be considered a suitable replacement for the Essen schedule.

Acknowledgment:
We wish to thank the staffs of the WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran. We are also grateful to Dr. Mohammad Mehdi Gouya for his administrative help and support.

Reprints:
Reprints will not be available from the author.

Funding:
This work was supported by grant from Pasteur Institute of Iran (grant number 1621).

Declaration of Interest:
The authors who have taken part in this study declare that they have nothing to disclose regarding the conflict of interest with respect to this manuscript.

References:

1. Lyles DS, Rupprecht CE. Rhabdoviridae. In: Knipe D, Howley P (eds) Fields virology, Philadelphia: Lippincott, Williams and Wilkins 2007; 1363-1408.

2. World Health Organization. Guide for rabies pre and post-exposure prophylaxis in humans. Department of Neglected Tropical Diseases-Neglected Zoonotic Diseases Team: 2010; http://www.who.int/rabies/PEP_prophylaxis_guidelines_June10.pdf.

3. Jackson AC . Human Rabies: a 2016 Update. Curr Infect Dis Rep 2016;18(11):38 (PMID: 27730539).

4. Bourhy H, Varsat AD, Hotez PJ, Salomon J. Rabies, still neglected after 125 years of vaccination. PLoS Negl Trop Dis 2010;4 (11):e839 (PMID: 21152052).

5. Warrell MJ. Current rabies vaccines and prophylaxis schedules: Preventing rabies before and after exposure. Travel Med Infect Dis 2012;10:1-15 (PMID: 22342356).

6. Rupprecht CE, Briggs D, Brown CM, et al. Evidence for a 4-dose vaccine schedule for human rabies post-exposure prophylaxis in previously non-vaccinated individuals. Vaccine 2009;27:7141-. (PMID: 19925944).

7. Ren J, Yao L, Sun J, Gong Z. Zagreb Regimen, an Abbreviated Intramuscular Schedule for Rabies Vaccination. Clin Vaccine Immunol 2015;22(1):1-5 (PMID: 25392012).

8. Hampson K, Cleaveland S, Briggs D. Evaluation of Cost-Effective Strategies for Rabies Post-Exposure Vaccination in Low-Income Countries. PLoS Negl Trop Dis 2011;5:e982 (PMID: 21408 121).

9. Wera E, Velthuis AG, Geong M, Hogeveen H. Costs of rabies control: An economic calculation method applied to flores island. PLoS One 2013;8:e83654 (PMID: 24386244).

10. Jackson AC, Warrell MJ, Rupprecht CE, et al. Management of rabies in humans. Clin Infect Dis 2003;36:6063. https://academic.oup.com/cid/article-abstract/36/1/60/283656

11. Rahimi P, Shirzadi MR, Farahtaj F, et al. Efficacy of Purified Vero Cell Rabies Vaccine (PVRV) under the Zagreb Regimen in Iran. Vaccine Research 2014;1(1):25-7 https://pdfs.semanticscholar.org/97fc/a4184c16c5b700fa53e09acadbbe0a9598c6.pdf

12. Mani RS, Madhusudana SN. Laboratory Diagnosis of Human Rabies: Recent Advances. Scientific World Journal 2013;2013: 569712 https://www.hindawi.com/journals/tswj/2013/569712/

13. Woldehiwet Z. Clinical laboratory advances in the detection of rabies virus. Clin Chim Acta 2005;351:49-63 (PMID: 15563871).

14. Durr S, Naïssengar S, Mindekem R, et al. Rabies diagnosis for developing countries. PLoS Negl Trop Dis 2008;26:e206 (PMID: 18365035).

15. Fooks AR, Johnson N, Freuling CM, et al. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. PLoS Negl Trop Dis 2009;3:e530 (PMID: 19787037).

16. Rahimi P, Vahabpour R, Aghasadeghi MR, et al. Neutralizing Antibody Response after Intramuscular Purified Vero Cell Rabies Vaccination (PVRV) in Iranian Patients with Specific Medical Conditions. PLoS One 2015 Oct 6;10(10):e0139171 (PMID: 26440665).

17. Gholami A, Fayaz A, Farahtaj F. Rabies in Iran: Past, Present and Future. J Med Microbiol Infec Dis 2014;2(1):1-10 https://jommid.pasteur.ac.ir/article-1-49-en.pdf

18. Ambrozaitis A, Laiskonis A, Balciuniene L, Banzhoff A, Malerczyk C. Rabies post-exposure prophylaxis vaccination with purified chick embryo cell vaccine (PCECV) and purified vero cell rabies vaccine (PVRV) in a four-site intradermal schedule (4-0-2-0-1-1): An immunogenic, cost-effective and practical regimen. Vaccine 2006;24:4116-4121 (PMID: 16545510).

19. Mahendra BJ, Narayana DA, Agarkhedkar S, et al. Comparative study on the immunogenicity and safety of a purified chick embryo cell rabies vaccine (PCECV) administered according to two different simulated post exposure intramuscular regimens (Zagreb versus Essen). Hum Vaccin Immunother 2015;11(2):428-34 (PMID: 25692792).

20. Ren J, Yao L, Sun J, Gong Z. Zagreb regimen, an abbreviated intramuscular schedule for rabies vaccination. Clin Vaccine Immunol 2015;22(1):1-5 (PMID: 25392012).

21. Liu H, Huang G, Tang Q, et al. The immunogenicity and safety of vaccination with purified Vero cell rabies vaccine (PVRV) in China under a 2-1-1 regimen. Hum Vaccin 2011;7(2):220-4 (PMID: 21311216).

22. Vodopija I, Sureau P, Smerdel S, et al. Interaction of rabies vaccine with human rabies immunoglobulin and reliability of a 2-1-1 schedule application for post-exposure treatment. Vaccine 1998; 6:283-6 (PMID: 3420976).

23. Lang J, Simanjuntak GH, Soerjosembodo S, Koesharyono C. Suppressant effect of human or equine rabies immunoglobulins on the immunogenicity of post-exposure rabies vaccination under the 2-1-1 regimen: a field trial in Indonesia. MAS054 Clinical Investigator Group. Bull World Health Organ 1998;76:491-5 (PMID: 9868840).

24. Huang G, Liu H, Tang Q, et al. Making rabies prophylaxis more economical: Immunogenicity and safety results from a preliminary study using a 2-1 intramuscular regimen in healthy volunteers. Hum Vaccin Immunother 2013;10(1):114-9 (PMID: 24008 819).