Evaluation of neonatal *Sprague-Dawley* rats as a potential animal model for neurovirulence test of an Iranian Mumps vaccine strain, RS-12.

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Abstract:

Mumps virus, a neurotropic member of paramyxoviridae, causes mumps disease. Since the 1960s, when the first live-attenuated vaccine against the mumps virus was developed, the mumps outbreaks have dramatically decreased. Monkey-based neurovirulence test has been developed and has been used to assess the safety of the attenuated mumps virus strains. However, laboratory and clinical findings have suggested that the monkey-based test may not necessarily reflect the neurovirulence behavior of the Mumps virus when administrated to the vaccinees. A neonatal rat-based MuV neurovirulence safety test has been developed and recommended by reference institutions in recent years. This test in Lewis rats was first introduced in 1998. This study aimed to evaluate the suitability of neonatal Sprague-Dawley rats for the neurovirulence test of an Iranian Mumps virus vaccine strain, RS-12. One-day-old Sprague-Dawley newborn rats were

intracranially injected with MRC-5 cell supernatant (assigned as “C” for control group), the RS-12 attenuated strain (assigned as “V” for vaccine group), and RS-12 wild-type strain (assigned as “W” for wild-type group) respectively. The animals were observed for 30 days post-injection regarding to the weight gain, viral titer in the brain tissue and appearance of hydrocephalus in the brain sections. The mean of weight gain in groups C and W was the highest and lowest respectively. Regression analysis of Log weight values revealed a significant difference between group C and group W. A significant difference between group V and group W was seen. There was no significant difference between the weight gain of group C and group V. No Mumps viruses were detected in the homogenized brain samples of group C, and in groups V and W, the viral titers showed a continuous decrease during the observation period. In the microscopic view of brain sections, the hydrocephalus started to form on day 15 post-injection and reached the highest extent on day 30th. On day 30 post-injection, the hydrocephalus area was determined as a maximum of 1%, 5%, and 10% for the C, V and W groups respectively. This study has introduced the newborn Sprague-Dawley rat model capable of demonstrating the neurovirulence potential of mumps viruses in vaccinees and distinguishing between wild-type and attenuated RS-12 strains. Further experiments are needed for optimization and validation of the test procedures.

Key words: Mumps Vaccine, Neurovirulence test, RS-12, Rat model.

1. Introduction:

Mumps is a highly contagious, vaccine-preventable disease caused by a paramyxovirus (1). The root of Mumps is obscure, but it is probably associated with an old English verb that means to grin, to grimace, or to mumble (2). The mumps virus (MuV) circulates between humans by direct contact through respiratory droplets and contaminated fomites (1). The disease occurs in 33% of unvaccinated people with no clinical signs (3). Non-specific symptoms such as anorexia, malaise, headache, and fever may occur, but the specific symptom is swelling of parotid glands. Less common consequences are oophoritis, orchitis, mastitis, and pancreatitis. More serious consequences of infection are aseptic meningitis and encephalitis, which are considered rare complications (1). In 40-50% of cases, particularly in children under five years old, mumps infection is associated with non-specific symptoms, particularly respiratory signs. Mumps infection is not the only causative agent of parotitis (4, 5). Immunity following natural mumps

infection is generally long lasting; however, re-infection may occur. In 75% of vaccinated individuals, mumps disease may occur with no clinical signs (1).

1.1. Infectious agent:

MuV has a non-segmented negative-sense RNA genome incorporated in an enveloped pleomorphic particle. MuV is classified in the genus *Orthorubulavirus* of the family *Paramyxoviridae* (1). MuV has only one serotype, but based on the nucleotide sequence of Small Hydrophobic (SH) and haemagglutinin-neuraminidase (HN) genes are classified into 12 genotypes (6, 7). The genome encodes seven proteins including nucleocapsid (N), phosphor (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins (6). Many cell types express MuV receptors, so the virus can enter into many cell types. However, Vero (African green monkey kidney cell) is widely used for virus isolation and propagation in laboratories (2). Although humans are the only natural hosts of MuV, various species such as monkeys, hamsters, mice, rats, and chicken embryos are susceptible to MuV infection (2). A newborn-rat model has been investigated for MuV neurovirulence in the last two decades, capable of use in preclinical neurotoxicology testing (for example in the assessment of vaccine safety) and to study the molecular basis of viral neurovirulence (2, 8).

1.2. Mumps vaccine history:

In 1934, a virus was identified as the etiological agent of mumps. MuV was first cultivated in chicken embryos by Habel and Enders in 1945. As the result of the successful cultivation of MuV in chicken embryos and cell cultures, an inactivated vaccine was developed in 1946 and tested in humans in 1951, but no longer administered because of the short period of immunity following vaccination. The first live attenuated vaccine was developed in the United States in the 1960s (9). Since then, worldwide administration of live-attenuated mumps vaccines has resulted in effective control and a dramatic decrease in mumps outbreaks (8). Inadequate cross-protection among MuV strains, when the formulated strain in the administered mumps vaccine is not from the same genotype of circulating MuV, could play a key role in the failure of global elimination of mumps (8).

1.3. Neurovirulence of MuV and the safety of mumps vaccines:

As a neurotropic and neurovirulent virus, MuV is capable of infecting the central nervous system in a proportion of mumps cases (8). All currently in-use mumps vaccines contain live-attenuated viruses (2), so it is essential to make sure about the safety of mumps vaccines (8). Mumps vaccine strains such as Jeryl Lynn, Leningrad-3, L-Zagreb, Urabe, etc. have been developed and certified for vaccine production during the last decades (9). Although it is rare, it has been reported that some vaccine strains such as (Leningrad and Urabe strains) may be capable of shedding from vaccinees and infecting unvaccinated or vaccinated individuals (10, 11). Therefore, to evaluate the neurovirulence and to assure the safety of mumps vaccines, neurovirulence tests should be carefully performed before starting clinical studies (8).

Mumps vaccines contain different live-attenuated strains, varying in immunogenicity, safety, efficacy, and adverse reaction profiles (12). Several studies have been conducted to evaluate these characteristics (9, 13, 14, 15, 16), providing the necessary information for national regulatory authorities to decide whether to manufacture and administer a mumps vaccine using a specific mumps virus strain or not (17).

The most common animal model for studying the neurovirulence of MuV is Rhesus Monkeys. However, it is well-documented that those mumps vaccines with acceptable safety profiles in the monkey models, may still cause meningitis and encephalitis in clinical use (8), and the neurovirulence test in a monkey model may not necessarily reflect the exact behavior of MuV in humans (18). Moreover, many authoritative organizations have put the suitability of monkey models for the evaluation of MuV neurovirulence under question (8). A neonatal rat-based MuV neurovirulence safety test has been developed and has been recommended by reference institutions in recent years. This model is much more convenient than monkey-based tests and can distinguish attenuated MuV strains from wild-type ones (8). The neonatal rat-based MuV neurovirulence test in Lewis rats was first introduced by Steven A. Rubin (8, 19) and gradually improved (20).

This study aimed to evaluate the suitability of neonatal Sprague-Dawley rats for the neurovirulence test of an Iranian MuV vaccine strain, RS-12. This was the first study on a neonatal rat-based MuV neurovirulence test in Iran.

2. Material and methods:

2.1. MuV, RS-12 strain: Both wild-type and attenuated strains were provided by the Human Viral Vaccines Department, Razi Vaccine & Serum Research Institute. Historically, the virus was

isolated from a clinically-approved mumps patient. The wild-type virus has adapted to the Human Diploid Cell line (MRC-5), following isolation and primary passages in Vero cells. The virus has attenuated using serial passages in MRC-5 cells (21).

2.2. Newborn rats: Sprague-Dawley newborn rats were provided by the Animal Husbandry Department, Razi Vaccine & Serum Research Institute.

2.3. Cell substrates: MRC-5 and Vero cells were provided by the Human Viral Vaccines Department, Razi Vaccine & Serum Research Institute.

2.4. Group assignments: Three groups of animals were assigned as C, V, and W. Group C (for control) contained 3 mothers and 26 newborn rats (C1-C3). Group V (for Vaccine strain-injected) contained 3 mothers and 37 neonatal rats (V1-V3). Group W (for wild-type-virus injected) contained 3 mothers and 34 neonatal rats (W1-W3). Each mother along with her newborn rats was kept in a dedicated cage.

2.5. Injection materials: One-day-old newborn rats in Group C, Group V, and Group W were injected with 20 microliters of MRC-5 cell supernatant, 20 microliters of the RS-12 attenuated (vaccine) strain sample containing $10^{3.5}$ viruses per ml, and RS-12 wild-type strain sample containing $10^{3.5}$ viruses per ml respectively.

2.6. Injection method: Sterile Hamilton syringes were used. The neonatal rats were gently fixed and subjected to intracranial injection in the left hemisphere, 2-3 mm in depth, at a location between the lambda and bregma regions. The injections were carried out under mild anesthesia.

2.7. Observation and sampling: The animals were observed for 30 days post-injection. All animals were weighed daily at 11:00 a.m, including a day before injection. Any unusual observations including deaths were recorded. On days 3, 6, 9, 12, 15, 19, 25, and 30 post-injection, an animal from each group was selected randomly. Following a deep anesthesia, the brains were carefully removed, a sagittal cut was made at the midline. The right hemispheres were homogenized in 1.5 ml DMEM (cell culture medium) and after centrifugation, the supernatants were frozen at -40°C for further virus titration. The left hemispheres were fixed in formaldehyde solution for further pathological evaluation.

2.8. Virus titration: The titer of MuV in the homogenized brain samples was determined using the Kurbur formula.

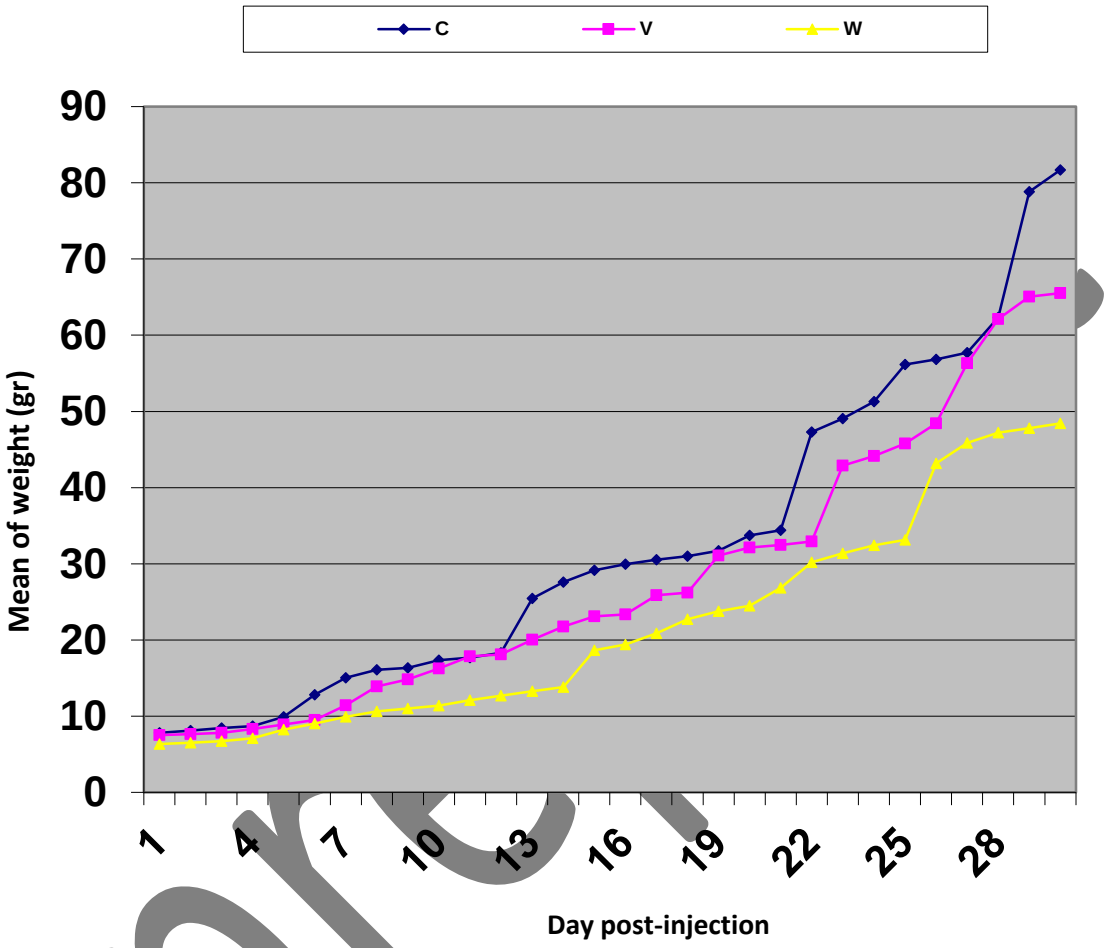
2.9. Pathological evaluation: Formalin-fixed, paraffin-embedded blocks were sectioned and stained using the Hematoxylin-Eosin method. The sections were microscopically observed for any pathological signs, particularly formation and the extent of hydrocephalus in the lateral ventricle.

3. Results:

The weight gain pattern, appearance of hydrocephalus in lateral ventricles, and the viral titer in the homogenized brain samples were followed as the main criteria of the neurovirulence test in a newborn rat-based model, according to the references.

The mean of weight gain in the C, V, and W groups showed a continuous increase during the 30 days of the observation period. The mean of weight gain in groups C and W was the highest and lowest, respectively. Despite group C, that the weight gain was still increasing at the end of the observation period, groups V and W had begun a stationary phase on day 28 post-injection (Figure 1). Regression analysis of Log weight values revealed a significant difference between group C and group W. There was also a significant difference between the group V and group W. There was no significant difference between the group C and group V.

Figure 1. Mean of weight gain of injected animals



163
164
165 The titer of MuV in homogenized brain samples is summarized in Table 1. Each sample was tested
166 3 times and the mean of calculated titers was considered as the viral titer. No MuV was detected
167 in the samples of group C. In groups V and W, the viral titers showed a continuous decrease during
168 the observation period.
169
170 **Table 1.** Viral titer in the homogenized brain tissues.

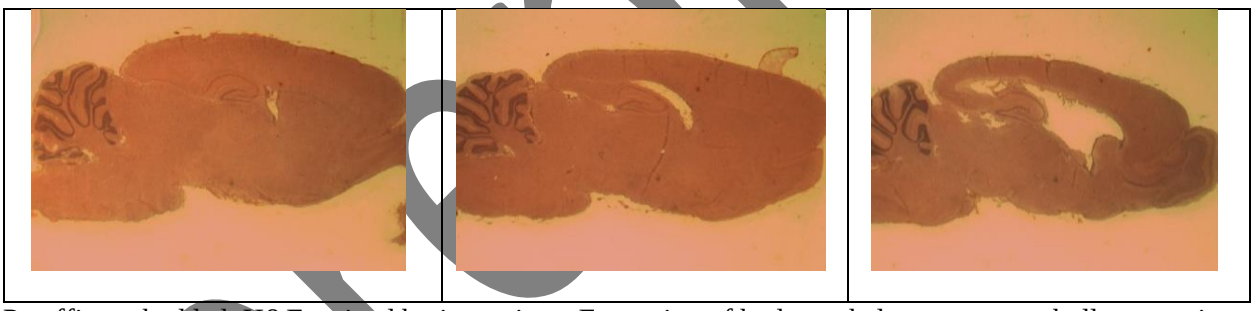
Mean of viral titer in the homogenized brain samples (-log CCID50/ml)

Day post-injection	3	6	9	12	15	19	25	30
Group C	ND	ND	ND	ND	ND	ND	ND	ND
Group V	3.25	3.17	3.00	3.08	3.00	2.75	2.50	ND
Group W	3.50	3.45	3.42	3.75	3.75	3.13	ND	ND

Titer of mumps virus during the 30-days observation period. Mean of three titrations are inserted as final titers. C: control, V: vaccine-injected group, W: wild-type-injected group, ND: not detected.

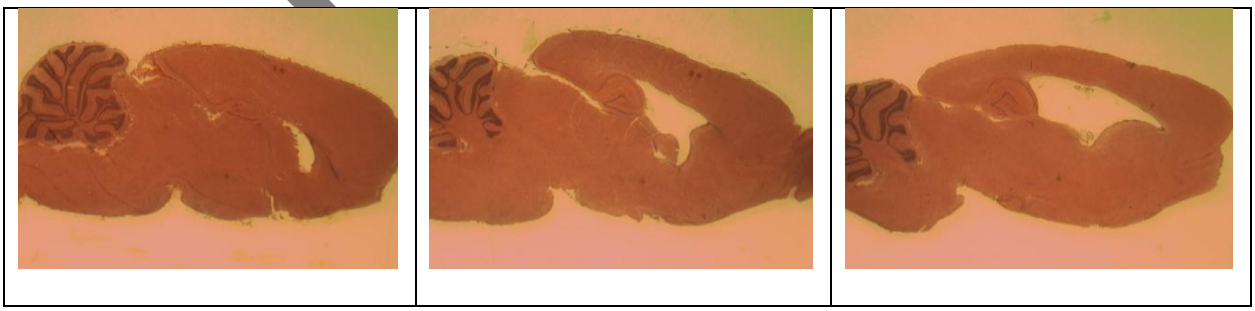
Sagittal sections were evaluated for the appearance of pathological signs, particularly hydrocephalus in the lateral ventricle. The hydrocephalus started to form on day 15 post-injection and reached to the highest extent on the day 30. To make the extent of hydrocephalus measurable, the area of the formed cavity in the lateral ventricle was compared against the whole brain section (excluding the conus and the optic lobe). At the end of day 30 post-injection, the hydrocephalus area was determined as a maximum of 1%, 5%, and 10% for the C, V and W groups respectively (Figures 2, 3).

Figure 2. Microscopic view of sagittal brain sections at day 25 post-injection.



Paraffin-embedded, H&E stained brain sections. Formation of hydrocephalus are seen as hollow area in the sections. Left: control group (injected with supernatant of MRC-5 cells), Middle: group V (injected with attenuated RS-12 mumps virus, and Right: group W (injected with wild-type RS-12 mumps virus).

Figure 3. Microscopic view of sagittal brain sections at day 30 post-injection.



Paraffin-embedded, H&E stained brain sections at the end of observation period. Formation of hydrocephalus are seen as hollow area in the sections. Left: control group (injected with supernatant of

MRC-5 cells), Middle: group V (injected with attenuated RS-12 mumps virus, and Right: group W (injected with wild-type RS-12 mumps virus).

4. Discussion:

Considering the neurotropic nature of MuV, the safety requirements of mumps vaccines should be carefully met by the manufacturers (22). One of the most important safety aspects of the mumps vaccine is the neurovirulence potential of the MuV vaccine strain in humans (19). These concerns have arisen when neurovirulence characteristics have appeared in some vaccinees (19). The risk of developing new live-attenuated mumps vaccines using strains with neurovirulence potential may persist unless an animal model becomes available with the ability to distinguish neurovirulent from non-neurovirulent strains (19).

At now, the standard method for assessing the neurovirulence risk of human vaccines, as recommended by the World Health Organization, is to test the vaccine's seed stocks in monkey models (19, 20, 22). However, the neurovirulence test of the mumps vaccine strains in monkeys (MNVT) is questionable in terms of the reliability of results (22), since the test is not sufficiently robust to predict the neurovirulence phenotype of MuV strains in humans (23). The clinical and pathological consequences of a vaccine strain of the MuV in monkeys do not necessarily reflect the neurovirulence of that strain in vaccinees (19). In other words, the MNVT test is not a true and accurate representation of the risk of neurovirulence in humans. According to the reports, the MNVT also cannot distinguish between wild-type and attenuated MuVs that are isolated from CSF in post-vaccination aseptic meningitis cases (22). There have been attempts to improve the efficacy of MNVT for accurate prediction of MuV neurovirulence in humans (23). It is therefore necessary to develop alternative animal models for evaluating the neurovirulence of MuV (22).

Attempts to introduce a murine model for MuV neurovirulence have been unsuccessful (22). Although the hamsters, as small animal models that are widely used in pathology studies, (19) have not been capable of reliably distinguishing neurovirulent strains from non-neurovirulent ones (8, 18). Moreover, studies on targeted mutagenesis with the aim of developing non-neurovirulent MuV strains have faced difficulties in the evaluation of efficiency due to the lack of a suitable animal model. Introducing an animal model that reliably predicts MuV neurovirulence in humans,

could also dramatically help to define the relation between molecular markers of MuV neurovirulence with greater certainty (19).

Successful attempts have been made over the years to introduce newborn rats as a reliable animal model of MuV neurovirulence (5, 19, 20, 22, 23). The early stages of these studies were conducted with an emphasis on the qualitative aspect, such that a highly neurovirulent strain called Kilham and a very harmless vaccine strain called Jeryl-Lynn were injected into the brains of newborn rats, and three important factors including the weight gain pattern, the viral titer in the brain tissue, and pathological signs in the brain sections have been considered as indicators of neurovirulence assessment (19). The results of this study showed differences in the process of weight gain, the incidence of hydrocephalus, as well as the ability to recover the virus from the animal's brain (19). As the next step, more strains of MuV have been included in the test and a scoring system for the severity of hydrocephalus has been established in the (hereafter called RNVT) test (23). Subsequent studies have also demonstrated the validation and reproducibility of the RNVT, and accurate prediction of the neurovirulence pattern of different strains of MuV (including wild, partially attenuated, or fully attenuated). Software has also been used to calculate the RNVT Score (20).

The main aim of this study was to evaluate the neurovirulence of wild-type and vaccine strains of an Iranian MuV, RS-12, in a newborn rat model. In the literature review, it was found that all RNVT tests were performed on Lewis rats. Since the Lewis breed was not available, it was decided to conduct this experiment using Sprague-Dawley newborn rats. This study was designed and conducted with a qualitative view to understand whether evaluation of neurovirulence criteria (weight gain pattern, formation of hydrocephalus, and recovery of MuV from the brain samples) following injection of RS-12 to Sprague-Dawley newborn rats is possible. Neither Sprague-Dawley newborn rats nor the RS-12 MuV strain had been examined in an RNVT before, so no data on the amount and viral titer suitable for injection to the brain were available. However, according to the methodology of a similar study (22), the volume of injection material per animal was adjusted for 20 microliters of viral samples containing $10^{3.5}$ particles/ml. The control group was injected with the same volume of MRC-5 cell supernatant, the same cell that had been used in the propagation of the RS-12 strain.

The weight gain curve of the C, V, and W groups showed the same pattern till day 5 post-injection but started to differ thereafter. Group C and Group W have experienced the lowest and highest

weight gains respectively. The difference between the weight gain of group V (that had been injected with the attenuated RS-12) and group W (that had been injected with Wild-type RS-12) was statistically significant. It means that regarding weight gain, Sprague-Dawley newborn rats can distinguish between wild-type and attenuated RS-12 viruses. These observations are fully consistent with the data reported in the corresponding articles.

Signs of hydrocephalus first appeared on day 15 post-injection and reached the highest degree on day 30th, when hydrocephalus was measured as much as 1%, 5%, and 10% in the C, V, and W groups respectively. In the relevant articles, the signs of hydrocephalus were observed on day 12 post-injection. This three-day delay may have a relation with the nature of the viral strain and the breed of animal in use. In the first RNVT study (19), there was also a report on signs of damage to the cerebellum on day 19 post-injection. Although in this study some abnormalities in the cerebellum's texture were seen in W and V groups on day 25 post-injection, we did not pay more attention to the cerebellum, since the same has been ignored in more recent studies. Based on the suggested scoring system, 0%, up to 6%, up to 12%, and up to 26% hydrocephalus has been considered as negative, mild, moderate, and severe respectively (23). Therefore, the grade of hydrocephalus in this study could be reported as mild for group V and moderate for group W. Regarding this finding, newborn Sprague-Dawley rats seem to be capable of distinguishing between wild-type and attenuated RS-12 strains. The pathogenesis of hydrocephalus caused by MuV is not well understood. However, it is proposed that the severity of hydrocephalus correlates with the ability of different strains of MuV to replicate in the rat's brain (23).

The mean of viral titer in the group W brain samples decreased till day 9 post-injection, then it increased and reached its maximum by the day 15 post-injection, followed by a decrease in a manner that the virus was not detected after day 19 post-injection. In the case of group V, the decrease in viral titer continued until day 25, when the virus become undetectable thereafter. These findings are consistent with similar studies (20, 24). However, there are slight differences that may be related to the differences between Lewis and Sprague-Dawley rats and the viral strain in use (RS-12). In addition, it should be noted that RS-12 wild-type and attenuated strains had been included in the current study, whereas a completely safe vaccine strain (Jeryl Lynn) were compared against a highly neurovirulent strain (Kilham) in the referenced study. Since the severity of the neurovirulence of wild RS-12 has not yet been compared with a highly neurovirulent strain such as Kilham, it cannot be expected to achieve the same results. However, isolating the virus from

the brain preparations (which represents the replication of the virus in the animal's brain) and obtaining similar results in terms of the co-incidence of the decrease of the viral titer and the development of neuropathological symptoms is very valuable and promising. Moreover, the fact that the attenuated virus has been isolated from the brain for a longer period compared to the wild-type one, may be attributed to the lower severity of brain damage in the animals injected with the attenuated virus.

This study has introduced a newborn Sprague-Dawley rat model capable of demonstrating the neurovirulence potential of mumps viruses in humans and distinguishing between wild-type and attenuated RS-12 strains. Further experiments are needed for optimization and validation of the test procedures.

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Author Contributions

Study concept and design: MK. Sh., M. T.

Acquisition of data: MK. Sh., M. T., A. M., MH. H., M. Sh.

Analysis and interpretation of data: MK. Sh., M. T.

Drafting of the manuscript: MK. Sh.

Critical revision of the manuscript: MK. Sh.

Statistical analysis: AR. Y.

Material support: R. Sh., A. F.

Study supervision: MK. Sh.

Ethics approval

The study was approved by Razi Vaccine & Serum Research Institute [Nº 2-18-18-89038], Karaj, Iran.

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Conflict of interests:

The authors declare that they have no conflict of interests.

Consent for publication:

All authors consent for open access publication.

Data Availability:

Not Applicable

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