Identification of some health indicators related to OIE notifiable viral diseases in Rainbow trout (*Oncorhynchus mykiss*) based on the strategic plan for producing Specific Pathogen Free (SPF) broodstock in Iran

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Abstract

In the year 2017, Iran had fisheries production amounted to 1 million tons, of which the total aquaculture was about 300 thousand tons that 160 thousand tons of aquaculture was regarding *Oncorhynchus mykiss*. Increase of fish stocking density in the pond results in escalation of the number of nutrients, the stresses and incidence of diseases. Among these diseases viral diseases could be identified in the field of rearing ponds, such as Infectious hematopoietic necrosis (IHN), Viral Hemorrhagic Septicemia (VHS) and Infectious Pancreatic Necrosis (IPN). Unlike other projects that sample is taken from fish tissues known as a sample, in this study each farm was selected as a research unit. According to the layout of the work based on different fields; the positive or negative result of the presence of the pathogens was recorded. However, aquatic samples were collected from Mazandaran province and Yasuj sites, located in north and southwest of Iran, respectively. Based on the results of Mazandaran area, it was revealed that the increase of bio-safety in the pre-broodstock farms did not have a significant difference ($p > 0.05$) compared with the increase of immunity level or serum antibodies. This index was relatively equal in fish of other areas. Contrarily, in the pre-broodstock farms, the lysozyme value as a non-specific immunity index had a significant increase, which indicates that the upgrading of the bio-safety level can reduce stress and shift the energy directly to the production.

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Introduction

In the year of 2017, Iran had fisheries production amounted to 1 million tons, of which the total aquaculture was about 300 thousand tons, and 160 thousand tons were related to Rainbow trout (Iran Fisheries Organization (IFO) 2018).

The earth's population increase leads to the rising demand for protein. Accordingly, a comprehensive request for fish and finfish products increased in aquaculture and fishery. Aquaculture production has been rising up to 64 million tons since 2012, exceeding beef meet in the last 2 years, and equaling fishery with an inconsistency around 90 million tons from 2000 (Jennings, Stentiford, Leocadio, Jeffery, Metcalfe, Katsiadaki, Auchterlonie, Mangi, Pinnegar & Ellis 2016).

Fish stress and diseases are the chief influences restricting spread out of aquaculture production. Epidemics and outbreaks of aquatic diseases have had a remarkable impression on the loss economics of the aquaculture industry. The FAO evaluated that approximately US $5 billion is annually mislaid due to the farm crop (Stentiford, Neil, Peeler, Shields, Small, Flegel, Vlak, Jones, Morado & Moss 2012).

Fish viral diseases are the most important results for growing up the stocking density in aquaculture production (Walker & Winton 2010). The pancreatic infectious necrosis virus (IPN) is the first fish virus that has been isolated from cell culture, and is still one of the fish viruses that most studies have been done on (Davies, McColl, Wang, Yu, Williams & Crane, 2010). The pancreatic infectious necrosis virus in its acute form can cause up to 100% mortality in young trout and has been known as one of the most important diseases associated with the salmon farming industry (Ahmadivand, Soltani, Behdani, Evensen, Alirahimi, Soltani, Hassanzadeh, Ashrafi-Helan 2018). Most fish that remain after the outbreak are carriers without symptoms. These carriers without symptoms can be the source of vertical transmission of the disease. The virus is present in the stool, especially under stress conditions such as spawning. The mortality ratio associated with the outbreak is completely variable from 5 to 100%, and various factors such as host, environmental and viral factors may affect the severity of the outbreak (Evensen & Santi 2008).

Infectious hematopoietic necrosis (IHN) virus is one of the three Rhabdovirus of fish that has been listed in the World Health Organization Animal Health Index (OIE 2010) with two other ones, VHS (viral hemorrhagic septicemia) and SVC (Spring Viraemia of Carp) (Dhar, Manna & Allnutt 2014). The predisposing factors of the disease include the species and age of the fish. Many viral diseases of the bony fish are more sensitive in the initial stage (younger) or lower stages of the disease (Jensen & Kristoffersen 2015).

Viral Hemorrhagic Septicemia (VHS) is one of the most dangerous and widespread viral diseases of fish, which was the first isolated from rainbow trout in European countries, in which it is one the most serious disease causing

outbreaks in rainbow trout (Wolf, 1988, Kim, Kim, Kim, Kim, Park, Kitamura, Kim, Kim, Han & Jung 2009). Viral Hemorrhagic Septicemia (VHS) is often known as causative mortality agent of Rainbow Trout, but also may cause deaths in other wild species of marine and freshwater (Gjevre, Ørpetveit, Tavornpanich & Lyngstad 2015).

According to the above mentioned references, the objective of this study was to concentrate on the isolation of three important viruses of the rainbow trout from the better management practice (BMP) farms of Iran to try to have the negative virus for SPF-spawner production.

Materials and Methods

In this study and according to the objectives of the paper, evaluation and monitoring of pathogens were widely studied during 2014-2015 but based on a retrospective study, in which the data of PCR and histopathology tests had been already collected. Based on the obtained data, the possibility of measuring the association between the probability of occurrence of disease and environmental and stress factors was analyzed. Finally, technical and specialized guidelines for controlling, preventing and eradicating diseases were selected on farms. In this regard, the fields were selected for review in coordination with other colleagues.

Sampling Methods

In addition to a sampling of fish tissues in this project, the field research was selected as the unit of research and according to the work arrangement based on the different fields; the positive or negative results of the presence of the pathogen were recorded. However, aquatic samples were collected in Tonekabon and Yasuj areas according to Table 1 (Histopathological examination), and after molecular tests (Table 2), the results of farms' contamination to each of the three viruses, the Viral Haemorrhagic Septicemia (VHS), the Infectious Pancreatic Necrosis (IPN) and Infectious Hematopoietic Necrosis (IHN) were obtained.

In Mazandaran province, the centers included the SPF broodstock site at the Cold Water Fish Research Center (located in the Dohezar area of Tonekabon), Farm G (located in the Sehezar area of Tonekabon), Farm F (located in Yasouj area) and Farm K (Gazanak area located close to Haraz River). The areas are listed in Table 1.

Table 1. Samples collected with rainbow trout fish tissue in different fields (Farms were introduced as abbreviations)

	Collected Sample	Center for sampling	Date of sampling
1	F., Internal organs	Yasuj	2016, fall
2	F., male, Internal organs	Yasuj	2016, fall
3	F., female, Internal organs	Yasuj	2016, fall
4	F., female, Internal organs	Yasuj	2016, fall
5	Tonekabon-K., female	Yasuj	2016, fall
6	Tonekabon, L., female	Yasuj	2016, fall
7	Tonekabon, K., female	Tonekabon	2016, fall
8	Tonekabon, L., male	Tonekabon	2016, fall

9	Tonekabon, N., Female	Tonekabon	2016, fall
10	Tonekabon, P., Female	Tonekabon	2016, fall
11	H., Female	Tonekabon	2016, fall
12	F., Male	Tonekabon	2016, fall
13	Tonekabon, N., Female	Tonekabon	2016, fall
14	Tonekabon, N., male	Tonekabon	2016, fall
15	F., eggs	Tonekabon	2016, fall
16	Tonekabon, P., eggs	Tonekabon	2016, fall
17	H., Female	Tonekabon	2016, fall
18	Tonekabon, P., Female	Tonekabon	2016, fall
19	Tonekabon,G.	Tonekabon	2016, fall
20	F., Female	Tonekabon	2016, fall
21	F., Female, eggs	Tonekabon	2016, fall
22	F., Male	Yasuj	2016, fall

Table 2. Sampling farms according to the cell culture protocols for *Oncorhynchus mykiss*

	Collected Sample	**Center for sampling**	**Date of sampling**
1	Seminal liquid	H.	Spring, late
2	Seminal liquid and fry	G.	Spring, late
3	F., Seminal liquid and eggs	Tonekabon	Spring, late
4	L., Male	Tonekabon	Summer, late
5	P., Female	Tonekabon	Spring, late
6	G., male, Seminal liquid and eggs	Tonekabon	Spring, late
7	N., Male	Tonekabon	Spring, late
8	F., Female Pre-broodstock	Tonekabon	Spring, late
9	F., internal organs, fry	Yasuj	Spring, late
10	VHSV	Anzali, Virus Lab	Re-culture
11	IPNV	Anzali, Virus Lab	Re-culture
12	IHNV	Anzali, Virus Lab	Re-culture

Result

The result of the Real-Time PCR replication in the obtained samples was negative using the selected primer for the IPN virus, and it seems that the disease did not exist in the selected farms.

Also, the replication of experimental samples from selected rainbow trout breeding farms using the IHN viral disease primer and the presence of a virulent virus in some experimental specimens, including Farm G in Road of 3000 (Male samples, Larvae and Sperm fluid) and farm H in Road of 3000 (Sperm fluid samples) has been confirmed.

The findings of VHS viral disease in experimental specimens of rainbow trout fish of different farms using one-step real-time experiment, Quanti Nova SYBR Green (Qia-Gen Germany), showed that samples of Yasuj (F) farm (Internal organs and pond of 4-4, female pre-broodstock, ovum and sperm), Farm G in Road of 3000 (Male, Larvae, and Sperm) and H farm in Road of 3000 (female fish) were infected with VHS virus.

The result of tissue samples of triple viral diseases isolated from selected farms for

Molecular biology and cell culture are available in Tables 3 and 4.

Table 3. Molecular biology assay on different tissue samples from selected farms

Row	Aggregated sample characterization	Sampling date	Test method	Disease
1	Yasuj (F farm) - Internal organs – Pond No. : 2-4	13th Nov. 2017	Manually	Negative
2	Yasuj (F farm) - Male sex - Internal organs - Pond No.: 1-4	13th Nov. 2017	Manually	Negative
3	Yasuj (F farm) - Female sex - Internal organs - Pond No.: 2-4	13th Nov. 2017	Manually	Negative
4	Yasuj (F farm) - Male sex - Internal organs - Pond No.: 1-5	13th Nov. 2017	Manually	Negative
5	Tonekabon- K farm-Female sex - No. 10501588	13th Nov. 2017	Manually	Negative
6	Tonekabon sample - L farm samples – No. 105000379	13th Nov. 2017	Manually	Negative
7	K farm-Female sex - No. 10501587- 10501587	Sep.- Oct. 2017	Manually	Negative
8	L farm samples – No. 10500887 and 10500379	Sep.- Oct. 2017	Manually	Negative
9	N farm- No. 10500932 and 10500133	Sep.- Oct. 2017	Manually	Negative
10	P farm - No. 10499743 and 10501083	Sep.- Oct. 2017	Manually	Negative
11	H farm, No. 10500142 and 10500256	Sep.- Oct. 2017	Manually	Negative
12	Yasuj (F farm) No. 10494571 and 10500929	Sep.- Oct. 2017	Manually	Negative
13	N farm- Female sex- No. 10495420	16th Nov. 2017	Manually	Negative
14	N farm - Male sex - No. 10500133	16th Nov. 2017	One-step method	Negative
15	Yasuj (F farm) - Female sex - Ovum – No. 10500929	16th Nov. 2017	One-step method	VHS
16	P farm, No. 10501083	16th Nov. 2017	One-step method	Negative
17	H farm, Female sex , No. 10500142	16th Nov. 2017	One-step method	VHS
18	P farm, Female sex, Number 10494947	16th Nov. 2017	One-step method	Negative
19	G farm, No. 190-96-VL	16th Nov. 2017	One-step method	IHN
20	Yasuj (F farm) - Female sex	16th Nov. 2017	One-step method	VHS
21	Yasuj (F farm) - Female sex Ovum	16th Nov. 2017	One-step method	VHS
22	Yasuj (F farm) - Male sex	20th Nov. 2016	One-step method	Negative

Table 4. Sampled farms from Rainbow trout fish cell cultures supernatants in different farms

Row	Aggregated sample characterization	Sampling date	Test method	The result of the disease
1	H farm, Sperm	20 June 2016	One - step Real-Time PCR	VHS- IHN
2	G farm - Fry and sperm	20 June 2016	One - step Real-Time PCR	IHN- VHS
3	Yasuj (F farm) - Ovum and sperm	20 June 2016	One - step Real-Time PCR	VHS-
4	L farm - Male sex	16th Nov. 2017	One - step Real-Time PCR	Negative
5	P farm - Male sex	16th Nov. 2017	One - step Real-Time PCR	Negative
6	G farm -Male sex, Fry and Sperm fluid	16th Nov. 2017	One - step Real-Time PCR	IHN
7	N farm - Male sex	16th Nov. 2017	One - step Real-Time PCR	Negative
8	Yasuj – Female Pre - broodstock	16th Nov. 2017	One - step Real-Time PCR	VHS-
9	Yasuj (F farm) - Internal organs – Ponds No.: 4-4, Male sex, Iranian fry (12)	20th Nov. 2016	One - step Real-Time PCR	VHS-
10	VHS Virus	Re-cultivating	One - step Real-Time PCR	Positive
11	IPN Virus	Re-cultivating	One - step Real-Time PCR	Unsuitable of the medium and, consequently, the negative result appeared
12	IHN Virus	Re-cultivating	One - step Real-Time PCR	Positive

Discussion

In this study, based on RT-PCR, seven healthy populations were diagnosed and transferred to the pre-quarantine center. Of course, farm N should be considered more thoroughly. Among the studied farms, three IHNV infected farms and one infected farm were identified as VHSV and IPNV which were removed from selected farms. Also, disease monitoring was performed in the quarantine center before reproduction, in the reproductive season and in the infected fish, and a VHS infected population was eliminated before the reproduction season began.

Based on the monitoring of farm F (Yasuj), VHS can be isolated from all internal organs, ovaries of female breeds, male and female breeder sperm (except in fry). Accordingly, the water temperature in the area was 13-15°C in the month of September, this is in line with the epidemic characteristics of the disease, which is a temperature of more than 10°C will cause a minimum of casualties, and the affected fish will carry the virus.

The virus has been isolated from farms G and H (Sperm and Fry) that were located in Mazandaran Province, which indicates more severe conditions (observed in fry).

Also, Faisal & Winters, 2011 reported that increased levels of Rainbow trout casualties caused by the VHS virus depend entirely on the age of fish, water temperature and fish species.

Regarding the incidence of the virus on each of the two G and H farms, we found that the IHN virus was isolated from these two centers only, indicating that the two centers were ready for the IHN outbreak. The most important part was the temperature above 10°C and this condition seems ideal for the onset of IHN.

No IPN virus isolates from any of the examined farms which could indicate the absence of contaminated eggs or broods transmitted to these ponds.

In another study on rainbow trout in Sardasht and Piranshahr, none of the three mentioned viruses were found. On the other hand, the study showed that the above-mentioned virus was positive and originated from Yasuj isolates.

In this research, N farm in the northwestern region of Iran was free from the three above mentioned viruses, which confirmed that this region is probably free from these three viruses, and therefore more research is needed, but in isolated samples from Yasuj, they had IPN and VHS viruses.

A comparison of these two studies suggests that VHS has been added to the body of water in Yasouj region during the past two years, which could lead to insufficient oversight of imported eyed eggs and breeders from other countries to Iran.

Also, Ahmadivand, Soltani, Mardani, Shokrpoor, Rahmati-Holasoo, Mokhtari and Hasanzadeh (2016) implied the presence of IPN in the waters of Mazandaran, which was not detected in the present study and could be due to the absence of a virus in imported samples or better control of veterinary or bio-security affairs in Mazandaran farms.

According to the Iranian Fisheries Organization (IFO), most Rainbow trout farms are importing eyed eggs from European countries, especially France. The largest number of VHSV isolates in France belonged to genotype I between 1971 and 1999 (Thiery, De Boisseson, Jeffroy, Castric, De Kinkelin & Benmansour, 2002), which mainly includes isolates from European freshwater fish farms and a wide variety of Marine species in the Baltic Sea (Einer-Jensen, Ahrens, Forsberg, Lorenzen 2004).

Also, the results of serum IgM levels in Mazandaran area showed that there was no significant difference between groups ($p < 0.05$). In this regard, the maximum serum immunoglobulin was recorded in farm K with 95.98 ± 5.44 without any difference to the amount of this indicator in farm G with high levels of 79.66 ± 5.92.

In terms of length and weight of fish, the fish from farm K, the SPF site and farm G had the highest length and weight. The results of lysozyme evaluation showed that the amount of lysozyme in the SPF site was significantly higher than the fish lysozyme in farm G ($p < 0.05$), although this difference was not significant ($p > 0.05$) compared to the fish lysozyme of farm K ($p > 0.05$) The number of lysozyme in the SPF site was larger than that of farm K fish.

The mean weight of the pre-broodstock farm was 1579.25 ± 81.69 which was larger than that of farm G fish with 1320.66 ± 65.07 range ($p < 0.05$), but the average weight of the farm was less than farm K which could possibly affect fish selection.

The amount of AST in farm K is higher than fish of other farms, especially farm G, and in confirmation of that amount, ALT has followed the same trend, Similarly, in another study, ALT values in the farm with more hygienic conditions were higher than the control group (Zorriehzahra, Hassan, Gholizadeh & Saidi 2010). Selecting fish from farm K, which had negative results of disease, was overlooked. In this study, the levels of hematocrit and hemoglobin in the fish of the pre-broodstock center were higher than that of the infected centers, and this finding was similar to the results of other researchers (Zorriehzahra *et al.*, 2010).

In rainbow trout fish, there is a high genetic variation for susceptibility to VHS (Henryon, Jokumsen, Berg, Lund, Pedersen, Olesen, Slierendrecht 2002, Henryon, Berg, Olesen, Kjær, Slierendrecht, Jokumsen & Lund 2005), but the age of the fish is more important. Younger fish are more susceptible to this disease. This is a similar result of the present study, which showed a higher incidence of VHS in larvae in selected farms of Mazandaran and especially Yasuj area. But in general, larger fish that have never been in contact with the disease in the past have experienced higher mortality rates when exposed to VHS.

VHSV transmission can be horizontal through contact with contaminated fish or water. Contaminated fish are known to be carriers over 6 months of age and viruses can be released by urine and reproductive fluids (Stepien, Pierce, Leaman, Niner & Shepherd

2015). In our study, the VHS virus was isolated from ovarian fluid and sperm.

Researchers found that VHS transmission can easily occur in the temperature range of 1 to15 °C, but it can also occur up to 20°C. Incubation time is dependent on the temperature and degree of the virus, and at higher temperatures, it takes between 5 and 12 days. The status of VHSV carriers in freshwater fish species is well known (Einer-Jensen *et al.* 2004).

The virologic status of such carriers depends on a wide range of parameters, including the duration of exposure and the geographical proximity to the fish pond outlet. The disease usually occurs at 4 to 14°C. At a temperature of 15 to 18°C, the disease usually occurs in a short period of time with relatively low cumulative mortality. At low water temperatures (1 to 5°C), the disease usually has high morbidity and low mortality rate, but high mortality rates also occur. VHS outbreaks occur throughout all seasons, but it is more common in the spring when the water temperature rises or fluctuates (Skall, Olesen & Mellergaard 2005).

In IPN disease, aging and lowering the temperature can lead to longer monition rates. The first sign of disease in the hatchery is the increase in sudden deaths in fry and fingerlings. The rotational state, with the head down and the tail to the top, are the hallmarks of the disease. Darkening, exophthalmia, abdominal swelling, bleeding in various areas of the body, pale gill, emaciation, and secretion of white pseudo-crystalline material from the rectum, which is more fragile than the fecal matter of IHN are more important clinical signs in affected fish.

In older fish, there is spotting around the gut and visceral haemorrhages (Ahmadivand *et al.* 2016). The transmission of the virus is mainly done horizontally in water and with the excretion of the virus from the urine (Einer-Jensen *et al.* 2004). There is no evidence of VHSV vertical transmission (Bovo 2005).

In the present study, based on the results of Mazandaran area, it was concluded that increased bio-security in the pre-broodstock farm had no significant difference ($p > 0.05$) on the increase in immunity and serum antibodies, and this indicator was relatively similar in fish from different centers. The lysozyme index as a non-specific safety index in the pre-broodstock farm had a significant increase, which may indicate that increasing the level of biological safety can reduce stress and change the direction of energy towards fattening. This is due to the increase in the weighted average and longitudinal mean of this center compared to other centers.

Since average weight of fish from farm K was less than other farms and because of higher amounts of AST, ALT and ALP of fish sampled from farm K, collecting pre-broodstock trout for SPF production plan from farm K should be prohibited.

High levels of some liver enzyms during the stocking on the pre-broodstock farm K could be indicated that the fish of this farm were under severe stress, therefore they could be susceptible to the OIE notifiable viral diseases, which need further study.

Nowadays, to prevent or minimize the occurrence of water-borne diseases, using herbal medicine, clean water intake or treated water, as well as good practice in hatcheries should be considered. (Kent, Buchner, Watral, Sanders, LaDu, Peterson & Tanguay 2011). The carrier fish could transmit the pathogen from infected fish to the egg vertically. Therefore, in such situation, the only way to ensure not to transmit the pathogen, is to produce fish completely free from the pathogen. SPF herding strategies can be the most important solution for controlling pathogen (Kent *et al.* 2011).

Conclusion and Suggestions

One of the best ways to cope with each of the 3 viral diseases mentioned above is to use heathy breeders selected by farmers, due to having higher level of non-specific immunity indices. To increase the level of immunity, use of vaccination can be an effective way of preventing the occurrence of diseases, provided that a governmental action is executed. Experiences have proven that none of these two ways can be succeeded individualy. Generally, healthy farm can prevent horizontal or vertical transmission of the disease. Major diseases could be caused by workers and nearby farms, or even non-expert visitors.

Performing intrusive inspection from the propagation farms, scheduled farm disinfection and eliminating contaminated breeders are the most effective measures in order to prevent occurrence of the diseases in the hatcheries.

Hatchery centers should have a bio-security program by the authorized organizations. According to the above, the arrival of live aquatic animals, including eyed-eggs from European or neighbor countries, requires more consideration either in borders or destination.

Conflict of interests

The authors declare that there is no conflict of interest.

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شناسایی برخی شاخص‌های بهداشتی مرتبط با بیماری‌های ویروسی قابل اخطار از نظر OIE در ماهیان قزل‌آلای رنگین کمان پرورشی بر اساس برنامه راهبردی تولید مولدین عاری از بیماری خاص (SPF) در ایران

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چکیده

در سال ۲۰۱۷، تولید شیلاتی ایران بالغ بر یک میلیون تن گردیده که از این میزان مقدار آبزی پروری حدود ۳۰۰ هزار تن بود که ۱۶۰ هزار تن آن مربوط به قزل‌آلای رنگین‌کمان می باشد. افزایش تراکم ذخیره ماهی در حوضچه‌های پرورشی باعث رشد مواد مغذی، استرس و بروز بیماری‌ها می‌شود. از جمله این بیماری‌ها می‌توان بیماری‌های ویروسی استخرهای پرورشی از جمله نکروز خونریزی عفونی (IHN)، سپتی سمی هموراژیک ویروسی (VHS) و نکروز پانکراس عفونی (IPN) نام برد. بر خلاف سایر پروژه‌هایی که نمونه‌ها از بافت ماهی گرفته شده‌اند، در این مطالعه مزرعه به عنوان واحد تحقیق انتخاب شده است. براساس چیدمان کار و زمینه‌های مختلف میدانی نتیجه مثبت یا منفی از حضور عوامل بیماری‌زا ثبت گردید. با این حال، نمونه‌های آبزی به ترتیب از استان مازندران و سایت‌های یاسوج در شمال و جنوب غربی ایران جمع‌آوری شدند. براساس نتایج حاصل از منطقه مازندران، مشخص شد که افزایش ایمنی زیستی در مزارع پیش مولد تفاوت معنی‌داری ($p> ۰/۰۵$) در مقایسه با افزایش سطح ایمنی یا آنتی بادی‌های سرم ندارد. این شاخص در ماهیان مناطق دیگر نسبتاً مشابه بود. برعکس، در مزارع پیش مولد، مقدار لیزوزیم به عنوان یک شاخص ایمنی غیر اختصاصی افزایش قابل توجهی داشته است. این امر نشان می‌دهد که ارتقاء سطح ایمنی زیستی می‌تواند استرس را کاهش داده و انرژی را مستقیماً به سمت تولید هدایت کند.

کلمات کلیدی: قزل‌آلای رنگین کمان، نکروز خونریزی عفونی، سپتی سمی هموراژیک ویروسی، نکروز پانکراس عفونی، ماهی عاری از بیماری خاص، ایران

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