A combined vaccine against *Brucella abortus* and infectious bovine rhinotracheitis

Govindasamy Kamaraj · Shankar R. Chinchkar · Lingala Rajendra · Villuppanoor Alwar Srinivasan

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Abstract The present study was undertaken to study the immune response in calves vaccinated with *Brucella abortus* strain 19, infectious bovine rhinotracheitis (IBR) vaccines in monovalent form and combined vaccine containing both antigen. The seroconversion of monovalent and combined vaccines was tested in seronegative cattle calves. IBR vaccine alone and combination with live *Brucella abortus* S19 vaccine elicited an anamnestic response on day 60 post booster but started declining from day 90 onwards against IBR. *B. abortus* S19 alone and in combination with IBR vaccine gave more than 2 log protection in mice two weeks post challenge. Fluorescence polarization assay analysis with sera samples of calves vaccinated with *B. abortus* S19 monovalent vaccine alone and in combination with IBR vaccine revealed the presence of *B. abortus* antibodies. The components of the combined vaccine did not show any evidence of interference in the development of immunity. This combined vaccine may provide economical and affordable biological for the control of brucellosis and IBR.

Keywords *Brucella abortus* S19 · Infectious bovine rhinotracheitis · Combined vaccine · Immune response · Protective index

Introduction

Sexually transmitted diseases of farm animals pose major health problems worldwide leading to economic losses. Among the several sexually transmitted pathogens *Brucella* sp. and bovine herpes virus-1 (BHV-1) most commonly found in bovine semen are capable of causing genital tract and respiratory disorders. Brucellosis, a major bacterial zoonotic disease with worldwide distribution caused by *Brucella abortus*, is a major cause of direct economical loss and major impediment for trade and export of animal and animal by-products [1]. Similarly, infectious bovine rhinotracheitis (IBR) a major viral disease of economic significance with widespread distribution in India caused by BHV-1 is manifested by infertility, abortions, early embryonic mortality, poor semen quality and respiratory tract disorders. It is needless to stress the importance of vaccinating cattle for establishing disease-free bull studs and thus maintain their effective reproductive performance.

Bovine brucellosis has been controlled by vaccination and eradicated by test and slaughter policy by many developed countries. However, this policy is not practical in a country like India due to socioeconomic reasons. Hence, vaccination appears to be the only tool available for effective control of this disease. One of the most widely used vaccines is an attenuated *B. abortus* strain 19 (S19) vaccine [2] which has been shown to induce protective immunity in cattle when administered during calthood. The control of IBR can be achieved by vaccination either with modified live or killed vaccine. Modified live vaccine, although effective, has the potential to shed virus in secretions and excretions which can act as a source of infection to susceptible animals. Betapropiolactone (BPL)-inactivated vaccine

G. Kamaraj · S. R. Chinchkar · L. Rajendra · V. A. Srinivasan (✉)
Indian Immunologicals Limited,
Hyderabad, Andhra Pradesh, India

E-mail: srini@indimmune.com

adjuvanted with aluminium hydroxide gel is therefore considered a potential alternative.

A bivalent vaccine is the tool of choice to protect against more than one sexually transmitted disease since it helps in reducing the severity of the disease in the herd and also animal manipulation, stress, labor and associated cost [3]. A combination vaccine consisting of live *B. abortus* S19 and BPL-inactivated BHV-1 antigens will be useful as a combination vaccine and such a combined vaccine will be cost-effective and also reduce the stress to the animals. Hence, a study has been undertaken with an objective of developing a combination vaccine for combating sexually transmitted diseases such as brucellosis and IBR.

Materials and methods

Bacterial strains and vaccine

The *B. abortus* S19 (vaccine strain) and *B. abortus* 544 biovar 1 (pathogenic strain) used in this study were obtained from the Animal Disease Research Laboratory (ADRL), National Dairy Development Board (NDDB), Anand, India. These strains were originally obtained from American Type Culture Collection (ATCC, USA) and maintained in ADRL, Anand according to propagation by standard procedure [4].

Brucella abortus S19 internal reference standard

B. abortus S19 vaccine produced by Indian Immunological Limited was used as an internal reference vaccine for potency testing in mice.

Brucella abortus antibody test kit

B. abortus Antibody Test Kit was procured from Diachemix Corporation, Wisconsin, USA. This kit was used to determine the presence of antibodies against *B. abortus* in bovine serum using fluorescence polarization assay method.

Cells and virus

Madin Darby bovine kidney (MDBK) cell line obtained from ATCC, USA and maintained as monolayer culture in the tissue culture laboratory of Indian Immunologicals Limited, Hyderabad was used for the propagation of BHV-1 virus. BHV-1 virus working seed banks obtained from ADRL, NDDB, India were used for production of BHV-1.

Mice

Five- to seven-week old Balb/C mice obtained from National Institute of Nutrition, Hyderabad were used for testing monovalent and combined vaccines.

Cattle calves

Thirty-five cross-bred cattle calves of either sex aged between 3 and 12 months seronegative for both *Brucella* and IBR antibodies were used in the vaccination experiments.

Brucella abortus S19 vaccine preparation

Freeze-dried vial of *B. abortus* strain 19 was rehydrated in sterile phosphate buffer saline (PBS; pH 6.4) and inoculated on potato infusion agar slopes. The slopes were incubated at 37°C for 72 h and the culture was washed with 5 ml of PBS pH 6.4 by rolling each tube gently between the palms. The harvest was transferred into the freeze-drying stabilizer medium aseptically and freeze-dried. Freeze-dried vials were stored at 4°C. Characterization of vaccine/seed was performed according to methods described by Alton [4]. Tests that discriminate *B. abortus* S19 from the reference *B. abortus* 544 biovar 1 strain were performed on tryptose agar (TA) plates supplemented either with penicillin G (2.5 IU/ml), erythritol (1 mg/ml) or thionin blue (2 µg/ml). The plates were incubated under 0% CO₂ and 5% CO₂ atmospheres for *B. abortus* S19 and Strain 544 respectively.

Bioreactor (Bioflow – 3000, New Brunswick Scientific, USA) with a working volume of 10 L was used for the bulk production of *B. abortus*. The *Brucella* strains in the culture broth were concentrated by microfiltration using 0.1 micron cassette. Purity was checked by inoculation in potato agar slope and dextrose Andrade's broth and gram's staining. The bacterial concentrate (3×10^{11}) was mixed with freeze-drying medium, filled in vials and freeze-dried. The freeze-dried vials were stored at 4–8°C until use.

Quality control tests on final vaccine

The number of viable *Brucella* strains per ml of the final bulk concentrate as well as final vaccine was determined essentially following a method described by Alton [4]. Test for dissociation (acriflavine test), identity and sterility were carried out as described by Alton [4]. The safety of *B. abortus* S19 vaccine was checked following the procedure described in OIE Manual [5]. Groups of 10 guinea-pigs each were given intramuscular injections of 1 dose of vaccine diluted in PBS, pH 6.4, known to contain 5×10^9 viable *Brucellae*. The vaccine passes the safety test if the inoculated animals do not exhibit adverse effects and mortality.

Fluorescence polarization (FP) assay

The fluorescence polarization (FP) assay was performed essentially following the method described by Nielsen

and Gall [6] using a fluorescence polarimeter (Diachemix LLC, USA). Briefly, 1 ml of diluted reaction buffer was pipetted into glass test tubes to which 10 μ l of negative control reagent was added. The solution was mixed well and the mixture was incubated at room temperature for 5 min. This mixture was used to blank the equipment. Then 10 μ l of *Brucella* FP conjugate was added and mixed. The mixture was then incubated at room temperature for 2 min. The reading expressed as millipolarization (mP) units was recorded. The above steps were repeated by sampling the negative control twice. The mean mP was calculated for the three replicates of the negative control. In the same way, the mean mP of positive control and samples were calculated.

IBR vaccine preparation

Production of IBR antigen

Roller culture bottles (Corning, USA) with 850 cm² surface area were used for growing the cells. Eagle's MEM with 10% adult bovine serum was used during growth of MDBK cells. MOI with 1:750 was used to infect the day-old confluent monolayer cells. Virus maintenance medium with 0.5% serum was used for the production lots. Twenty lots were prepared with each lot size containing 20 $\times$ 850 cm² flasks. Bottles showing more than 90% cytopathic effect (CPE) were frozen and after two cycles of freezing and thawing the virus was harvested. Virus cultures from each lot were pooled and centrifuged at 2500 rpm for 15 min to clarify the virus. A sample from pooled virus harvest was tested for virus infectivity titer. The clarified virus culture supernatant was inactivated using BPL (Ferak, Berlin GmbH:NMR 98.5%) at 1:500 at 37°C for 90 min and the pH was adjusted to 7.2 to 7.4.

Formulation of monovalent IBR vaccine

Inactivated IBR vaccine with aluminium hydroxide gel (CZ Veterinaria, Spain) was prepared using an antigen concentrate which had a virus titer of $10^{8.37}$ TCID₅₀ / ml before inactivation. Aluminium hydroxide gel (10 mg per dose) was used as an adjuvant in the vaccine formulation.

Formulation of combined IBR and *B. abortus* vaccine

B. abortus S19, live freeze-dried vaccine (5 dose vial) was rehydrated by mixing 10 ml of the inactivated IBR Algel vaccine and 2 ml of the mixture was used as a single dose.

Viral infectivity assay

Ten-fold dilutions of the virus were prepared and viral infectivity was determined by titration of the virus in

microtiter plates using MDBK cells. The virus titer was calculated using method described by Spearman and Karber [7] and expressed as log₁₀ TCID₅₀ / ml.

Inactivation/sterility test

Virus inactivation/sterility test was carried out using 2 $\times$ 150 cm² bottles with confluent MDBK monolayer cells. After removal of spent media 10 ml concentrated (ten-fold) and inactivated virus was inoculated in to the flasks and kept at 37°C for 30 min. Subsequently 30 ml of Eagle's MEM containing 0.5% adult bovine serum was added to each flask and incubated at 37°C for 4 days. After 4 days, the supernatant was harvested and inoculated into fresh bottles containing MDBK monolayer cells. The bottles were observed for 4 days for CPE. The process was repeated thrice. Complete inactivation was indicated by the absence of CPE in three consecutive passages in MDBK monolayer cells.

Enzyme-linked immunosorbent assay

The Chekit-trachitest (Idexx Laboratories, Switzerland) serum-screening enzyme immunoassay (EIA) kit was used for detecting antibodies against BHV-1.

Microserum neutralization test (MNT)

Neutralizing antibody titer against the vaccine virus was estimated in MNT by the constant virus-varying serum dilution with 100 TCID₅₀ of virus against two-fold serum dilutions.

Safety test

Two susceptible calves were inoculated 2X dose of each monovalent and bivalent vaccine by subcutaneous route. They were observed daily for local as well as systemic reactions for a period of 7 days.

Immunogenicity studies in mice

The potency of *B. abortus* S19, monovalent and the bivalent vaccine containing *B. abortus* S19 and IBR were tested in mice following the method described in OIE Manual [5]. The mice were bled before challenge and their sera separated and pooled and the micro neutralization test (MNT) was carried out to determine the IBR specific seroconversion from IBR, IBR + *B. abortus* S19 group.

Immunization studies in cattle calves

Three groups consisting of 10 calves each at 3–12 months of age were used for the experiment. The first group (Group A) received 2 ml of IBR (inactivated) monovalent Algel

vaccine by subcutaneous route on day '0' followed by a booster on day 28. The animals were bled on day '0' and on day 28 after primary immunization and on day 30, 60, 90, 120 and 150 after booster immunization. The sera samples were analyzed by MNT to determine BHV-1 neutralizing antibodies.

The second group (Group B) received 2 ml of *B. abortus* S19 vaccine (50×10^9 cfu) by subcutaneous route. The animals were bled on day 0, 28, 60, 90, 120, 150 and 180 after primary immunization. The sera samples were analyzed by FPA to study the antibody response to vaccine.

The third group (Group C) received 2 ml of *B. abortus* S19, live and IBR, inactivated combined vaccine by subcutaneous route. The primary immunized animals were boosted with IBR (inactivated) vaccine on day 28. The vaccinated animals were monitored twice daily for rise in body temperature and local tissue reaction. The animals were bled on day 0 and on 28 after priming and on day 30, 60, 90, 120 and 150 days after booster immunization. The sera samples were analyzed by MNT for anti-IBR antibodies and FPA to determine response to *B. abortus* S19 vaccine.

Five calves of similar age group were kept as unvaccinated controls.

Statistical analysis

The micro neutralization titer values obtained for the anti-IBR antibodies against monovalent and combined vaccines were analyzed for statistical significance using Student's 't' test.

Results

The seed organism was inhibited by thionin blue, penicillin G and *i*-erythritol and did not require CO₂ for growth. Organism in bioreactor revealed the presence of gram-negative bacteria. Cultural and biochemical characteristics also confirmed to *B. abortus* S 19 in the antigen concentrate.

The freeze-dried vaccine was subjected to quality control (QC) check to determine the viable *Brucellae* count which was found to be 2.6 to 2.8×10^{11} cfu per 5 doses. The *Brucellae* when subjected to acriflavine test showed the

intactness of the colonies and lack of dissociation. Gram's staining revealed the presence of gram-negative coccobacilli arranged either singly or in pairs. No acid and gas production was observed on day 2, 4 and 7 post-inoculation when sterility test was carried out using dextrose Andrade's broth. Further subculturing on day 3 by spreading the inoculated broth on potato infusion agar slope and incubation for 5 days at 37°C revealed colonies characteristic of *Brucella*. The vaccine when subjected to guinea pig inoculation did not result in adverse effects or mortality thereby indicating that the vaccine was safe.

The inactivated IBR vaccine passed the safety test as indicated by the absence of local and systemic reaction in calves. The vaccine also passed the sterility test. The results of potency testing of *B. abortus* S19 monovalent and combined vaccines prepared with inactivated IBR antigen in mice are shown in Table 1. *B. abortus* S19, monovalent and combined vaccine *B. abortus* S19 + IBR gave more than 2 log protection two weeks post challenge. Potency of monovalent *B. abortus* S19 and S19 + IBR did not differ significantly.

The IBRV antibody titer in the sera of mice vaccinated with IBR monovalent and *B. abortus* S19 + IBR vaccine as determined by MNT were 4 and 6 respectively. Serological response in mice to monovalent and combined formulation of IBR with *B. abortus* S19 did not differ significantly.

Inactivated IBR monovalent vaccine formulations were tested in 10 cattle calves (Group A) and antibody responses on days 28, 60, 90, 120 and 150 days post-vaccination were good. Antibody titers of 8 and 118 were recorded on 28 dpv and 30 days after booster vaccination respectively. The antibody level declined to 24 on 180 dpv (Fig. 2). There was no difference in the immune response towards IBR monovalent and combined vaccines ($P > 0.05$).

B. abortus S19 vaccine was tested in 10 cattle calves (Group B) and the antibody response was analyzed by FPA (Fig. 3) after different periods of vaccination. All the vaccinated animals were seropositive on day 28 post primary vaccination and on days 60, 90, 120 and 150 post-vaccination and FPA values declined on 180 dpv.

B. abortus S19 and IBR combined vaccine was tested in 10 cattle calves (Group C). All animals in this group were

Table 1 Mice potency results of *Brucella* vaccines (monovalent live *B. abortus* S19 and combined vaccine prepared using live *B. abortus* S19 and inactivated IBR antigen)

Vaccines	Mean cfu of <i>B. abortus</i> S544		Protective Index
	Control group	Vaccinated group	
<i>B. abortus</i> S19 monovalent internal reference vaccine	4.54	1.52	3.02
<i>B. abortus</i> S19 monovalent vaccine	4.54	1.21	3.33
<i>B. abortus</i> S19 and IBR combined vaccine	4.54	1.88	2.66

administered with a bivalent vaccine followed by booster with IBR vaccine alone on 28 dpv. The results of the antibody titer in calves vaccinated with bivalent vaccine on days 0, 28, 60, 90, 120, 150, and 180 post-vaccination are given in Table 2 and Fig. 3. Booster response for IBR observed was evidenced by a sharp rise in antibody response (121) 30 dpv but fallen to 82, 43, 28 and 20 on days 90, 120, 150 and 180 post-vaccination respectively. Results of FPA indicated that all the vaccinated animals were seropositive on 28 days post primary vaccination and up to 150 dpv to *B. abortus* S19 vaccine and FPA values declined on 180 dpv. None of the animals examined showed local reaction to the vaccine and the body temperatures were normal.

All the five unvaccinated control calves remained seronegative to *B. abortus* and IBR through out the duration of the experiment.

The Chekit-trachitest (Idexx Laboratories, Switzerland) serum-screening enzyme immunoassay (EIA) kit performed for detecting antibodies against BHV-1 showed that the day 0 samples i.e. before vaccination were seronegative while the post vaccination samples, were positive results up to day 180 post vaccination indicating the seroconversion towards vaccination against BHV-1.

Discussion

Genital infections of livestock cause severe productivity losses. Two important pathogens, *B. abortus* and IBR virus,

colonize the genital tract of cattle and buffalos and cause serious reproductive disorders. In India, prophylactic vaccination appears to be the only solution for control of these two diseases as culling and slaughter of animal is impossible due to socioeconomic reasons. Combination vaccines are considered as a boon to veterinarians and farmers since it saves labor, cost and reduces stress to the animals. We made an attempt to study the effects of combining *B. abortus* S19 live vaccine with inactivated IBR vaccine.

Bacteriological differentiation of *B. abortus* S19 vaccine strain from *B. abortus* biovar 1 field strains was achieved by its lack of CO₂ requirement for growth and its sensitivity to 5 IU/ml of penicillin G, 1 mg/ml of erythritol and 2 µg/ml of thionin blue [4]. The vaccine strain tested was found to meet all the prescribed characteristics. The sensitivity to *i*-erythritol is considered as important differential characteristic of S19, and has been widely used for differentiating the vaccine strain from *B. abortus* biovar 1 field strains. The results of biochemical characterization concur with that of Alton [4].

In this study we used the monovalent *B. abortus* S19 as a lyophilized preparation which had a live bacterial count of 2.6 to 2.8 × 10¹¹ cfu per 5 doses. Freeze drying losses were minimal, i.e. 7% to 9%. The freeze-dried vaccine complied with OIE standards for dissociation, identity and sterility when it was subjected to various quality control tests [5]. The freeze-dried vaccine passed the potency, safety and purity tests as per OIE Manual [5].

Fig. 1 Potency results of *Brucella* vaccines (monovalent and combined vaccine prepared with live *B. abortus* S19 and inactivated IBR antigen) in mice

Table 2 Serum antibody titers in cattle calves to IBR vaccines (monovalent and combined vaccine with *B. abortus* S19) by micro neutralization test (dpv – days post vaccination)

Groups	Animal No.	0 dpv	28 dpv	60 dpv	90 dpv	120 dpv	150 dpv	180 dpv
Group A (IBR (inactivated) monovalent vaccine)*	5247	<2	8	32	32	32	16	16
	4893	<2	8	128	64	64	32	32
	4973	<2	8	256	128	64	64	32
	5236	<2	8	64	64	32	32	32
	4598	<2	4	128	64	32	32	16
	4463	<2	8	64	32	32	16	16
	4563	<2	8	64	64	32	32	32
	4676	<2	8	64	32	32	16	16
	4782	<2	8	128	64	32	32	16
	4474	<2	16	256	128	128	64	32
	Mean	<2	8	118	67	48	34	24
Group C (IBR (inactivated) + <i>Brucella abortus</i> S19 bivalent vaccine)*	354	<2	8	64	32	16	16	16
	531	<2	8	128	64	64	32	32
	510	<2	4	128	128	64	32	16
	376	<2	4	32	32	16	16	8
	502	<2	8	32	16	16	8	8
	529	<2	8	64	32	16	16	8
	378	<2	8	128	64	64	32	16
	385	<2	16	256	256	64	64	64
	327	<2	16	256	128	64	32	16
	384	<2	8	128	64	32	32	16
	Mean	<2	9	121	82	43	28	20
Unvaccinated controls	451	<2	<2	<2	<2	<2	<2	<2
	486	<2	<2	<2	<2	<2	<2	<2
	492	<2	<2	<2	<2	<2	<2	<2
	506	<2	<2	<2	<2	<2	<2	<2
	509	<2	<2	<2	<2	<2	<2	<2
	Mean	<2	<2	<2	<2	<2	<2	<2

* The mean antibody titers between the two groups did not differ significantly; $P>0.05$

The protective index of *B. abortus* S19, monovalent and combined vaccine *B. abortus* S19 reconstituted with IBR inactivated vaccine were 3.33 and 2.66 respectively. As per the OIE Manual [5] the test vaccine is declared satisfactory if the immunogenicity value obtained in mice is significantly lower than that obtained in the unvaccinated controls and does not differ significantly from that obtained in mice vaccinated with the reference vaccine. The reference vaccine had a protective index of 3.02. The results indicate that the vaccine meets the specification of immunogenicity test prescribed in the OIE Manual [5].

In the first group (Group A), seroconversion was estimated in each animal against IBR virus by MNT and

enzyme-linked immunosorbent assay (ELISA). The ELISA results showed that the animals became seroconverted by day 21 post vaccination and remained ELISA positive until day 180 post vaccination. Serum neutralizing antibody titers of all vaccinated animals were well above 8 on 28 days post-vaccination. Schipper and Kelling [8] reported that none of the calves vaccinated with inactivated IBR vaccine exhibited a response based on serum titer 28 days following the initial vaccination. Following a booster vaccination, 8.3% of the calves had titers of 8. But the present study did not concur with their findings.

The results obtained in this study are in agreement with the data reported by Kilari et al. [9]. Booster vaccination

after the primary dose resulted in serum antibody titers as high as 256 in 20% of the animals. At three months post-vaccination, 20% of the vaccinated animals showed titers more than 64, which appeared to be maintained consistently during the study period. At 180th day post-vaccination 100% of the sera showed titers ranging from 16 to 32. These findings corroborate with the findings of Kilari et al. [9] who reported that at 180 days post-vaccination, majority of the vaccinated animals showed titers ranging from 8 to 16.

In second group (Group B) lyophilized *B. abortus* S 19 vaccine was tested in cattle calves and seroconversion was

Fig. 2 Serological response in cattle calves to IBR vaccines (monovalent and combined vaccine with live *B. abortus* S19) by micro neutralization test

studied using FPA. Sero monitoring was performed at different time points such as 0, 28, 60, 90, 120, 150 and 180 days post-vaccination. Cattle calves tested by FPA prior to vaccination with *B. abortus* S19 vaccine were seronegative for *Brucella* antibodies. All the cattle calves vaccinated with S19 elicited positive response 28 days after vaccination. All the animals remained seropositive during 2nd to 5th month post-vaccination. On 180 dpv, 40% of the animals showed less than optimal response. These results indicate that cattle vaccinated with S19 vaccine produce antibodies that can be detected by FPA. The findings of the present study corroborate with the results of Stevens et al. [10] who reported that cattle generally had positive or suspect response during 2 to 10 weeks after vaccination with S19.

In the third group (Group C), *B. abortus* S19, live and inactivated IBR combined vaccine was tested in seronegative cattle calves. Mean serum neutralizing antibody titers against IBR in all the vaccinated animals were well above 8 on day 28 post-vaccination. The ELISA results showed that the animals became seroconverted by day 21 post vaccination and remained ELISA positive until day 180 post vaccination. A booster vaccination after priming resulted in serum antibody titers as high as 256 in 20% animals. While the remaining animal had 32 to 128 SN₅₀ values. Antibody titers against IBR virus declined gradually on the 180dpv as seen in IBR inactivated, monovalent vaccinated group. All the calves seroconverted positive response on day 28 post-vaccination against *B. abortus* S19. All the animals maintained positive antibody titers from 2nd to 5th month post-vaccination. Sixty percent of animals remained sero-

Fig. 3 Serological response in cattle calves *B. abortus* S19 monovalent and combined vaccine (*B. abortus* S19 + IBR) by fluorescence polarization assay

positive on day 180 post-vaccination. The results of this study are in accordance with the findings of Aguirre et al. [11] who reported a maximum of 68.5% of FPA positive animals at week 4 with a slow decline.

Current *B. abortus* vaccine formulation is a lyophilized vaccine preparation containing 2×10^{11} cfu of *B. abortus* S19 sufficient for 5 doses. The results of the present study indicated that *B. abortus* freeze-dried vaccine can be combined with IBR vaccine. Antibody responses to both the antigens were satisfactory which showed that there is no antigenic competition. Serological response to *B. abortus* and IBR antigen in animals vaccinated with bivalent did not differ significantly ($P > 0.05$) from the serological response in groups of calves vaccinated either with *B. abortus* S19 or IBR vaccine.

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