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## Original article

# Development and production of rabies virus-specific monoclonal antibodies and evaluation of their neutralizing and protective potentials

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## ABSTRACT

**Background:** Rabies is a severe viral infection that causes acute encephalomyelitis and has a case fatality rate of almost 100%. There is no cure for symptomatic rabies, but post-exposure prophylaxis (PEP), which comprises vaccines and anti-rabies immunoglobulins (RIGs), can successfully prevent the development of irreversible clinical symptoms. In a situation with low resources, the primary PEP protocol confronts significant access and implementation challenges that could be successfully overcome using RIGs instead of monoclonal antibodies (mAbs). The current study focuses on the important characteristics of mAbs against rabies that are currently under development and emphasizes their potential as a cutting-edge therapeutic strategy. **Methods:** BALB/c mice were vaccinated using immunizing Freund's adjuvanted emulsions of the inactivated purified Vero cell rabies vaccine (PVRV, VERORAB) made by Aventis Pasteur. The created hybrids were subjected to an ELISA 12 days after fusion to check for the presence of antibodies specific to the rabies virus. **Results and conclusion:** There are 4 adequately formed murine hybridomas that secrete mAbs that are specific to the rabies virus. These 4 stable hybrids, designated 1E4, 1E9, 2F3, and 4E1, were successfully cloned into four stable clones. The specificity of the generated hybrids was validated using the western blot. The effectiveness of the mAbs cocktail made from the 4 hybridomas was assessed along with the neutralizing capacity of the generated mAbs. Mice challenged with 1000 LD50 of the rabies virus strain were completely protected by the mAbs cocktail given to them 24 hours after infection, while all control mice contracted the disease.

## Introduction

Rabies disease is an acute viral infection of the central nervous system caused by the rabies virus (RABV). This disease remains an important public health problem and has a long history that dates back to antiquity [1, 2, 3]. Except for a few rare instances where the rabies vaccination was given before the development of clinical disease, treatment efforts to

date in humans have been ineffective for this acute, swiftly deadly neurological disease [4].

Rabies virus is a single-stranded, negative-sense, unsegmented, encapsulated RNA virus with dimensions of roughly 60nm x 180nm. It is made up of a transmembrane glycoprotein spike-covered lipid bilayer on the outside and a protein nucleocapsid, or internal protein core that contains the nucleic acid [5]. It belongs to the family

Rhabdoviridae and the genus *Lyssavirus*. It is a cunning neurotropic pathogen and causes top-priority neglected tropical diseases in the developing world. Nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and multifunctional polymerase protein (L) are the five proteins that the viral genomic RNA codes for. The primary surface antigens to which neutralizing antibodies are attached are glycoproteins. Neurological symptoms are brought on by the glycoprotein antigen's binding to acetylcholine receptors, which results in a disturbance of neurotransmitter release [6].

By biting infected mammals, the rabies virus is spread through the saliva of those animals. Despite being nearly always preventable, rabies is a serious illness that affects humans worldwide. Its fatality rate is approximately twelfth among all infectious diseases [2]. Normally, this virus spreads from dog to dog through bite wounds and the saliva of the sick animal, which contains the virus [7]. It has occasionally been suggested that non-bite transmission can occur through ingestion, inhalation, and other mucosal exposure. Mouth-licking and eating food that has been regurgitated have both been linked to infection; both behaviors are widespread among social candidates. These transmission methods might have been present during epidemics [8].

Behavioral abnormalities brought on by rabies infection in the brain are probably caused by limbic region neuronal infection, which makes rabies transmission by biting easier. The parasympathetic nervous system, which is in charge of infecting the salivary glands, skin, heart, and several other organs, plays a key role in the centrifugal spread of the rabies virus away from the central nervous system (CNS). The saliva of rabies vectors secretes the infectious rabies virus, which is crucial for transmission to other hosts [2, 9, 10].

Post-exposure prophylaxis, which involves the simultaneous delivery of a rabies vaccine and RIG, has the potential to prevent lethal rabies virus infection [11]. Human RIG (HRIG) and equine RIG (ERIG), both produced from pooled sera of human donors and horses immunized against rabies, respectively, are the two forms of RIGs that are employed. MAbs that neutralize the RABV provide the chance to replace these hyper-immune serum preparations, which is a well-acknowledged necessity [12, 13, 14].

By merging the appropriate cells, a spleen cell with the genetic information to manufacture particular antibodies and a selectable myeloma cell that was adapted to continuous and strong growth in culture, the first mAb of predetermined specificity was created [15]. The production of mAbs was driven by the requirement for homogeneous antibodies to serve as repeatable reagents. They have the obvious advantages of a single specificity. Polyclonal antibodies are heterogeneous mixtures of molecules with a variety of binding affinities that make up a small portion of the complex mixture of serum proteins [16].

Mouse mAbs, as well as human mAbs, have been shown to protect rodents from a lethal RABV challenge [14, 17, 18]. One of the most potent human mAbs, SO57, which manifests strong activity against a variety of RABV strains, was described by [19].

Neutralizing antibodies were most effective early in the course of the infection, before the invasion of the brain system, according to the Wistar Institute experiments. The effectiveness of mAbs is debatable once the neurological system has been infiltrated. Rabies mAbs have definite advantages over the current treatments. They may be produced with great precision, and it is simple to keep an eye on their quality. One antibody could not be employed alone due to the likelihood of escape variations, so Wistar's scientists and the WHO advised selecting mAbs with various specificities and combining them into a "cocktail." [10]. The mAbs have shown their action in several animal models, and as technology advances, it may be easier to produce them in large quantities at low cost and with easier quality control than polyclonal serum [10]. Currently, antibodies are very promising candidates that are widely utilized, and incorporated in a variety and wide range of antigen detection and infectious diseases' diagnostic [20, 21, 22], prophylactic [23], and therapeutic [24] protocols. The aim of the present work was the preparation of RABV-specific mAbs, their characterization, and the evaluation of their neutralizing and protective potentials as a post-exposure prophylaxis strategy.

## Materials and Methods

### Ethics approval and consent to participate

According to the (Animal Research: Reporting of In-Vivo Experiments-ARRIVE) standards, the current study is reported. The Institutional Animal Care and Use Committee's

(IACUC) rules were strictly adhered to throughout the current study's protocols involving the use of animals. The IACUC approval number given to the current protocol is Vet CU 03162023686.

### Statistical analysis

All data obtained in this study were collected, classified, tabulated, and analyzed using MICROSOFT OFFICE 365 EXCEL SPREADSHEET features.

### Experimental animals

Ten BALB/c mice (8 weeks old, 25-30g) from the experimental animals' farm, VACSERA, Ministry of Health, Egypt. These mice were healthy and were kept under good environmental and nutritional conditions. The BALB/c mice were divided into 2 groups, the first one contains eight BALB/c mice that were used for immunization with the rabies virus vaccine for the production of highly immunized splenocytes. The second group was composed of 2 adult mice that were used for the preparation of peritoneal macrophages that were used as feeder cells.

Twelve Swiss albino mice (6 weeks old, 10-12g) were obtained from a laboratory animal's farm, VACSERA, Ministry of Health, Egypt. These mice were used for the challenge experiment.

### Virus

The source of the rabies virus incorporated as a vaccinal strain in the current study was the inactivated rabies virus vaccine: which is a freeze-dried purified rabies virus vaccine Pasteur virus Rabies virus (PVRV), VERORAB, WISTAR strain RABIES PM/WI 38-1503-3M grown on Vero cells produced by Aventis Pasteur.

Another rabies virus strain was supplied by the Rabies Research Department (RRD) in VACSERA, Ministry of Health, Egypt. This strain was isolated previously in the VACSERA laboratory from the brains of rabid dogs. This strain was stored as infected mouse brains at  $-75^{\circ}\text{C}$  after titration by mice inoculation.

### Cell lines

A myeloma cell line (Plasmacytoma), the P3-NS1 cell line was used. These myeloma cells synthesize but do not secrete the K light chain. It was kindly provided by the Central Laboratory for Monoclonal Antibody Production (CLMAP), VACSERA, Egypt.

### Other materials

-Antibody enzyme conjugates (THERMO-FISHER) for ELISA: Peroxidase conjugated Anti-

mouse polyvalent immunoglobulins (IgG, IgA, IgM) the affinity isolated antigen-specific antibodies developed in goats using purified mouse IgG, IgA, and IgM as immunogens. Alkaline phosphatase-conjugated anti-mouse IgG (whole molecule) antibody developed in goats. The isolated antigen-specific antibody was adsorbed with rat serum proteins and stored at  $2-8^{\circ}\text{C}$ .

-Enzyme substrate (THERMO-FISHER) for horse radish peroxidase: 2, 2 azino-bis (3-ethyl benzthiazoline-6-sulphonic acid-ABTS).

-Adjuvants (SIGMA-ALDRICH): Two types were used, namely, complete Freund's adjuvant (CFA) which was used only for the priming immunizing dose, and incomplete Freund's adjuvant (IFA) which was used for booster immunization.

-Polyethylene glycol (PEG 1500 & 50%) (SIGMA-ALDRICH): Sterile-filtered, endotoxin, and hybridoma tested. It is used as a fusogenic material in fusion between splenocytes and myeloma to give hybrids.

-Hypoxanthine-Aminopterin-Thymidine (HAT) Medium Supplement 50X (SIGMA-ALDRICH): Selective medium for hybridomas (HAT medium supplement 50X) lyophilized,  $\gamma$ -irradiated powder for use in aseptic procedures and cell culture tested and stored dry at  $-20^{\circ}\text{C}$ .

-Hypoxanthine-Thymidine (HT) Medium Supplement 50X (SIGMA-ALDRICH): another media used as a transition medium for HAT-selected hybridomas before transfer to RPMI medium. (HT medium supplement 50X) lyophilized,  $\gamma$ -irradiated powder for use in aseptic procedures and cell culture tested stored dry at  $-20^{\circ}\text{C}$ .

-OPI Medium Supplement: Lyophilized,  $\gamma$ -irradiated powder for use in aseptic procedures and cell culture tested, endotoxin-free. Used in the preparation of cloning media with HT medium supplement. Stored dry at  $-20^{\circ}\text{C}$ .

-Dimethyl sulphoxide (DMSO) research grade (SERVA): It is used as a freezing medium for cells (sterile filtered, endotoxin, and hybridoma tested).

Preparation of a stable emulsion of purified rabies virus vaccine (PVRV-VERORAB) in Freund's adjuvants

Mice were immunized with an antigen that was prepared for injection by emulsified antigen. An emulsion was prepared from rabies virus vaccinal antigen and an equal volume of Freund's adjuvant. The stability test was done by dropping the emulsion

into a beaker containing water. A stable emulsion was an oil-in-water emulsion, which would not disperse when dropped into the water and kept in the refrigerator for 24 hours.

### **Immunization protocol to produce immune spleen cells**

Before vaccination, the pre-immune serum must be collected to serve as a baseline control for antibody screening. A capillary tube is used to collect 100–200 µl of blood after the mouse has been bled by cutting around 1–2 mm at the tip of its tail, the blood sampling step was repeated throughout the whole immunization protocol before each immunization day. From this blood, the serum is extracted and can be cryo-preserved [25]. Six successive doses of the rabies virus vaccine (PVRV-VERORAB) antigens were used [26, 27]. Each dose was injected into the mice according to the following scheme:

-First priming dose: An emulsion containing equal volumes of rabies virus vaccinal antigens (35µg of the rabies vaccine proteins) and complete Freund's adjuvant was injected I/P in mice using a dose of 0.3 ml/mouse.

-Second booster dose: Two weeks after the first immunization dose, an emulsion containing equal volumes of rabies virus vaccinal antigen emulsified in incomplete Freund's adjuvant was injected I/P in the immunized mice using 0.3 ml/mouse.

-Third, fourth, and fifth booster doses: These booster doses were done exactly like the second dose at 2-week intervals.

-Final booster dose: Four days before fusion, 0.3 ml of the rabies virus vaccine alone without adjuvant was injected I/P.

Screening of mice for antibody production was performed using ELISA, confirmed high titer antibodies were the sign of giving the final booster dose as well as proceeding to the next step

### **Preparation of myeloma cells for fusion with splenocytes from immunized BALB/c mice**

To assure their sensitivity to the HAT selection medium used following cell fusion, myeloma cells are immortalized and grown with 8-azaguanine. The myeloma cells are cultured on 8-azaguanine for one week before cell fusion. High vitality and rapid proliferation are required in cells. Only the merged cells can survive in culture when

using the HAT media. The myeloma cells were maintained in 250 ml tissue culture flasks in 50ml of complete Roswell Park Memorial Institute (RPMI)-1640 culture medium with 20% fetal calf serum (FCS) under environmental conditions of 5% CO<sub>2</sub>, 37°C, and 98% relative humidity incubator [28]. The density of the cells was between 10<sup>7</sup>-10<sup>8</sup> cells/ml. Before fusion, the P3-NS1 myeloma cells were in the log phase of growth (i.e., over 95% of cells were viable).

### **Preparation of splenocytes from immunized BALB/c mice**

Fusing antibody-producing spleen cells, which have a limited life span, with cells derived from an immortal tumor of lymphocytes (Myeloma) results in a hybridoma that is capable of unlimited growth. Briefly, four days after the last immunization, the titer of anti-rabies virus-specific antibodies was measured by ELISA, and the high-responder mouse was selected. The selected mouse was sacrificed, the spleen was removed, and minced and the splenocytes were harvested [29].

### **Polyethylene glycol (PEG) aided fusion**

The splenocytes were suspended in RPMI media supplemented with 20% FCS. The tube was then centrifuged at 1500 rpm for 5 minutes and re-suspended in 5ml RPMI supplemented with 20% FCS. The harvested spleen cells and the myeloma cells were counted and adjusted to a ratio of 10:1, respectively. The spleen cells were added to the myeloma in a centrifugation tube and then pelleted together. The pelleted cells were washed with 10 ml serum-free medium in a centrifugation tube and the supernatant was discarded. To the packed cells 1ml 50% PEG 1500 was added drop by drop over 1 min with tube rotation in the hand [30]. The tube was then put in a water bath at 37°C for 1 min. with gentle shaking. After centrifugation at 100xg, which is equal to 700 rpm for 1 min at 37°C, 1ml RPMI medium without serum was added drop by drop over 1 min with gentle agitation. Another 4.5 ml RPMI medium without serum was added drop by drop over 3 min with gentle agitation. Five ml RPMI medium without serum was added over 2 min with gentle agitation. Then RPMI medium without serum was added to reach a total volume of 50 ml. This was followed by centrifugation at 200xg for 5 min at 37 °C, the supernatant was discarded, and the cell pellet was gently re-suspended in a 9 ml complete RPMI culture medium. The cell mixture was distributed in three 4-tissue culture plates (240 wells). The outside

wells of the plates were filled only with RPMI medium. In the first tissue culture plate, the cell suspension was distributed in 100 µl/well). Six ml of complete RPMI culture medium was added to the cell suspension, mixed, and distributed in the second plate. For the third plate, 3 ml complete RPMI culture medium was added, and the cells were mixed and distributed in the fourth plate. The plates were left in a 5% CO<sub>2</sub>, 37°C incubator overnight before adding HAT medium.

#### **Hypoxanthine-Aminopterin-Thymidine selection**

On the second day after fusion, HAT medium was added to the 96-well plates. HAT medium (2 ml HAT + 48 ml RPMI) was added to replace the old medium in half (2X). On days 4, 6, and 8, as well as every two to three days after that until the first screen, this medium was replaced with HAT medium (1 ml HAT + 49 ml RPMI) (1X). All the myeloma cells perished within three to four days, and starting around the fifth day, hybrids started to show up. By using ELISA, specific antibody production (at days 12–14) was monitored. At least three days passed between the screening and the last medium change [29]. Positive antibody-secreting wells were chosen and extended after the screening.

#### **Expansion of culture and treatment of the cells with Hypoxanthine-Thymidine medium before cloning**

Using 24 wells tissue culture plate 1 ml HT medium was added to each 2 ml well. The cells in fusion plate-positive wells were suspended and 100 µl/well was transferred to a 2 ml well and mixed, then 100 µl of this cell mixture was returned to the original well in the fusion plate. Two days later much supernatant was removed as possible, and a fresh HT medium was added [31]. When cells were nearly confluent (up to about one week) the new wells and the original wells were re-assayed for rabies-specific antibodies using ELISA.

#### **Cloning, sub-cloning, isotyping, and cryopreservation of the prepared hybridomas**

Hybridoma cells that were secreting mAbs that were specific to the rabies virus were then cloned using limiting dilution, multiplied, and kept in liquid nitrogen [30]. Small groups of hybridoma cells from 96-well plates were cultivated in tissue culture before being selected for antigen binding and cloned afterward. Cloning through "limiting dilution" ensures that most wells each contain no

more than one clone. Careful judgment was required to choose hybridomas that can expand as opposed to completely losing the cell fusion product due to under-population or insufficient in vitro growth at high dilution. In rare cases, the produced antibodies would be harmful to the delicate cells kept in vitro [32, 33, 34].

Using the mouse immunoglobulin isotyping kits (Mouse Typer Isotyping panel, Bio-Rad, Hercules, CA, USA) according to the manufacturer instructions, and the agar gel precipitation test, to identify the class of the generated specific mAbs [35].

#### **Evaluation of the neutralizing potential of the prepared mAbs**

The protecting capability and neutralizing potential of the anti-rabies mAbs cocktail prepared from the 4 recovered hybridomas were determined in a mice challenge experiment with a rabies virus strain supplied by the rabies research department in VACSERA, Ministry of Health, Egypt. For the challenge experiments, twelve 6-week-old Swiss albino mice (10–12g) were utilized, which were procured from VACSERA Laboratory Animal Farm, VACSERA, Ministry of Health, Egypt. The rabies virus strain 1000 LD<sub>50</sub> was produced as 10% mouse brain homogenate in sterile phosphate-buffered saline (PBS) and utilized in the challenge trials as follows; Six mice were given 0.1 ml of a virus suspension containing 1000 LD<sub>50</sub> of the virus intramuscularly in the hind leg. After 24 hours post-infection, the mice were injected with 1.5 IU/0.1 mL of the mAbs cocktail at the same leg. The rabies virus was administered to the second batch of six mice in the same manner, and at the same intervals after infection, 0.1 mL of sterile saline was injected to be used as a "placebo" model. The recommended and internationally standardized dose of ERIG for passive immunization of humans (40 IU/kg body weight) was used to calculate the mAbs dose [36].

#### **Results**

##### **Determination of the protein content of the rabies virus vaccine used for immunization**

The total protein content of the rabies virus vaccine used for immunization of BALB/c mice was 8 mg/ml as measured with the Biuret test.

##### **Monitoring of immunization of BALB/c mice with rabies vaccine**

Two weeks after the third vaccination, the eight inoculated mice were bled, and serum was drawn. The generated anti-rabies antibody titer

against the rabies vaccine was evaluated using ELISA. Although several mice displayed a positive immunological response, mouse number 6 was chosen for the fusion experiment because of their stronger response (**Table 1**).

#### **Fusion of myeloma cells with rabies-immunized spleen lymphocytes and microscopic examination of the fusion plates**

The three fusion plates (240 wells) were microscopically examined twelve days after fusion. The hybrid cell growth that covered 10% to 50% of the wells' surface area was chosen. Only 76 wells (32%) of the 240 wells (four plates) under examination exhibited hybrid growth (**Table 2**).

#### **Examination of the tissue culture supernatants from wells showing hybrid growth for anti-rabies antibodies**

Twelve days after fusion, the first ELISA test was performed on supernatants from the hybrid cell development, which covers 10% to 50% of the surface area of the wells. Five days before screening, the medium-changing process was stopped in these wells. Out of 240 total wells, only 14 (5.8%) were positive (**Table 3**).

#### **Number of stable hybrids producing rabies virus-specific monoclonal antibodies**

To continue the cell growth, the positive wells were chosen and moved to 24 well plates. After being propagated in 24 well plates, only four hybrids demonstrated stability; the other 10 hybrids perished. The locations of the positive stable hybrids were given.

#### **Cloning of positive hybridomas using the limiting dilution method**

To find pure hybrid cells expressing monoclonal antibodies against the rabies virus, positive recovered hybridomas were cloned using the limited dilution approach. Cloning was mostly done to separate positive hybridoma from non-producing cells. To identify the wells that had a single clone that produced anti-rabies monoclonal antibodies, the cloning plates were inspected under a microscope and serologically using ELISA.

#### **Screening of the cloned hybridomas using ELISA**

The hybridomas were successfully cloned via indirect ELISA, and four stable clones that produced mAbs specific to the rabies virus were found: namely 1E4, 1E9, 2F3, and 4E1. Four of the acquired clones had positive results from a confirmatory western blot assay on the supernatant of the clones.

#### **Isotyping of the monoclonal antibodies produced by the four clones**

All four of the produced clones were identified and found to be of the IgM isotype using the agar gel method and mouse immunoglobulin isotyping kits.

#### **The neutralizing potential and protective efficacy of the mAbs cocktail prepared from the 4 developed hybridomas**

Mice challenged with 1000 LD50 of rabies virus were completely protected by the mAbs cocktail made from the 4 hybridomas, while all challenged control mice acquired the clinical presentation of the disease.

**Table 1.** Measurement of anti-rabies polyclonal antibodies in serum of BALB/c mice immunized with rabies vaccine using ELISA.

Immunized mice	Titer of anti-rabies antibodies							
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
	Results were expressed as optical density reading OD							
Negative control	0.511	0.500	0.443	0.296	0.281	0.144	0.106	0.063
Mice 1	1.717 +	1.434 +	1.082 +	0.731 +	0.391 +	0.114 -	0.058 -	0.039 -
Mice 2	1.801 +	1.466 +	1.132 +	0.772 +	0.402 +	0.149 ±	0.106 -	0.001 -
Mice 3	1.737 +	1.432 +	1.088 +	0.696 +	0.396 +	0.120 -	0.094 -	0.010 -
Mice 4	1.622 +	1.338 +	1.045 +	0.682 +	0.369 +	0.097 -	0.044 -	0.056 -
Mice 5	0.651 +	0.616 +	0.588 +	0.569 +	0.544 +	0.439 +	0.365 +	0.257 +
Mice 6	1.663 +	1.602 +	1.582 +	1.580 +	1.570 +	1.440 +	1.395 +	1.218 +
Mice 7	0.559 +	0.511 +	0.480 +	0.431 +	0.354 +	0.287 +	0.191 +	0.017 -
Mice 8	1.162 +	1.160 +	1.139 +	1.074 +	0.975 +	0.884 +	0.695 +	0.451 +

**Table 2.** Results of microscopic examination of fusion plates 12 days post-fusion.

Plate number	Total wells number	Wells showing growth		Wells showing dead cells	
		No.	%	No.	%
1	60	41	68	19	32
2	60	17	28	43	72
3	60	12	20	48	80
4	60	6	10	54	90
Total	240	76	32	164	68

**Table 3.** Results of screening the fusion plate for anti-rabies antibodies producing hybridoma using ELISA 12 days post fusion.

Plate number	Total wells number	Wells showing positive hybrids		Wells showing negative hybrids	
		No.	%	No.	%
1	60	3	5	57	95
2	60	2	3.33	58	96.67
3	60	5	8.33	55	91.67
4	60	4	6.67	56	93.33
Total	240	14	5.8	226	94.2

## Discussion

The rabies virus, which may infect a wide variety of warm-blooded species, causes rabies, an acute viral infection of the central nervous system. Even though it is virtually always prevented, rabies nonetheless ranks as the eighth most lethal of all infectious diseases in terms of fatality in humans.

Behavioral abnormalities brought on by rabies infection in the brain are probably caused by limbic region neuronal infection, which makes rabies transmission by biting easier. Centrifugal propagation of the rabies virus occurs along neural pathways, particularly in the parasympathetic nervous system, which is in charge of infecting the heart, skin, salivary glands, and a number of other organs. The saliva of rabies vectors secretes the infectious rabies virus, which is crucial for transmission to other hosts [9].

Post-exposure prophylaxis, which involves the simultaneous delivery of a rabies vaccination and rabies immune globulin, prevents lethal rabies RIG. HRIG and ERIG, [37] both produced from pooled sera of human donors and horses immunized against the rabies vaccine, respectively, are the two forms of RIGs that are employed [38]. MAbs that neutralize the rabies virus (RV) provide the chance to replace these hyper-immune serum preparations, which is a generally acknowledged necessity [39].

By combining the appropriate cells, a spleen cell with the genetic information to make specific antibodies and a selectable myeloma cell that was adapted to continuous and strong growth in culture, [15] produced the first monoclonal antibody of specified specificity. Although mAbs are replacing the production of polyclonal antisera because they offer significant advantages in terms of specificity, potency, reproducibility, and freedom from contaminants, polyclonal antibodies have been used successfully for more than a century to treat and prevent the spread of infectious diseases [40]. The monoclonal antibody production technology, on the other hand, is one of the fastest-growing areas of the biotechnology and biopharmaceutical industry. It is anticipated that this technology will account for more than 60% of the entire biopharmaceutical industry by 2030. More than 200 mAbs have been approved for therapeutic purposes [41], and several hundred are approved for diagnostic approaches.

Over polyclonal antibodies, the development of monoclonal antibodies against the

rabies virus offers significant benefits. It is very precise, has a low propensity to trigger an allergic reaction known as a hypersensitive reaction, and its manufacturing is tightly under control. The mAbs' quality can be easily checked, and they can be produced in a lab rather than a farm as is the case with polyclonal antibodies [42]. Because the potential for escape variations prevented the use of a single antibody, Wistar's scientists adopted the tactic of selecting mAbs with various specificities and mixing them to create a "cocktail" [43].

In the current study, peritoneal macrophages were added to the tissue culture plates before the fusion operation to serve as feeder cells and support the formation of the hybridoma. This is in line with the work done by [44], even though other types of feeder cells, such as thymocytes, may be used. When used as feeder cells, macrophages likely secrete growth factors that promote the growth of hybridomas, remove toxic byproducts from media, and have a synergistic effect. Additionally, it significantly raises the output of hybrids and cleans the cultures of dead parental cells and detritus [30].

The splenocytes, B-cells, taken from hyper-immunized BALB/c mice were fused with myeloma cell lines out of worry for the fusion operation (P3NS1). The log-phase myeloma cells with a viability of over 90% were chosen. The PEG 1500, 40% concentration encouraged the fusion of B-cells and myeloma cells. PEG is a polywax substance that encourages cell adhesion and nuclei exchange. It promotes the development of somatic cell hybrids and enables an enhanced and streamlined method for cell fusion in vitro [30]. Normally cell fusion can occur spontaneously in the culture at low levels, but its incidence can be increased by treatment of cells with a fusogenic agent like PEG [45, 46, 47]. Most laboratories still widely use this agent in infusion technology at a concentration of 40-50% (W/V). Several researchers have experimented with various myeloma cell-to-splenocyte ratios in fusion technology. The cell ratio between splenocytes and myeloma in the current study was 10:1. In a technique similar to this, [48] merged ( $10^8$ ) spleen cells and ( $10^7$ ) myeloma cells while 50% PEG 1000 solution was added to the mixture of the two cells.

Using hypoxanthine aminopterin thymidine, spleen-myeloma cell hybridomas were created, which were chosen from the unfused cells



and unwanted fused cells (HAT selective medium) according to [46]. Fusion cultures were exposed to a selective medium containing hypoxanthine, aminopterin, and thymidine, resulting in less work and a higher yield. Due to the random nature of cell fusion, the cell culture contains a variety of myeloma-spleen, myeloma-myeloma, and spleen-spleen fusions. Myeloma-spleen cells fuse only when the cell combination is cultured in HAT media. Hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme necessary for the incorporation of hypoxanthine, is absent in a Plasmacytoma cell line [30].

Post-fusion care started seven days after plating out the cells in the HAT medium with the replacement of half the medium with fresh medium containing HT, instead of HAT. At this time, small hybridoma growth was detected at the margin of several wells. Supernatants from these wells were collected and tested for the presence of mAbs using ELISA [45]. In the present work, using an ELISA, antibodies against the rabies virus were checked in hybridoma cell development that covered 10% to 50% of the surface area of the wells 12 days after fusion. Most scientists employ ELISA to check for monoclonal antibodies made by hybridomas [45, 49].

Four positive hybridomas were successfully recovered producing specific monoclonal antibodies against the rabies virus. Successful cloning was achieved with these hybridoma cell lines. Utilizing the limited dilution procedure, the positive hybridoma was cloned to produce a single stable clone that produced particular monoclonal antibodies against the rabies virus. To eliminate the non-producing cells from the clones and prevent the overlap of the antibody-producing cells with the non-producing ones, cloning was carried out twice. Additionally, to ensure that the antibody-producing cell is monoclonal (i.e., to confirm its derivation from a single hybrid cell). This agrees with that stated by [14, 46].

#### **Study limitations**

Rabies mAbs have a great need in medicine and show promise for improving public health, but gaining clearance and adoption is a difficult process. For decades, researchers have been searching for rabies mAbs to complement or replace blood-derived RIG, but only one product has received approval, and three others are presently undergoing clinical trials. There are other explanations for this

slow progress, such as the fact that rabies is mostly a disease of developing nations and that businesses may be reluctant to engage in expensive product development due to anticipated low returns. Additionally, it is possible that the clinical development pathway may be difficult given the mortality rate of rabies and the logistical and ethical challenges associated with evaluating this susceptible group of exposed persons.

#### **Conclusion**

The prepared rabies virus-specific mAbs cocktail from the 4 cloned hybridomas, which are of IgM isotypes, proved effective in inducing 100% protection against rabies virus infection when given to rabies virus-challenged mice 24 hours post-infection as compared with control-challenged non-treated mice, which died from rabies challenge with rabies virus.

#### **Conflict of interest:**

None declared.

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