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Research Article

Preliminary Assessment of Newly Developed *Escherichia coli* Mastitis Vaccines in Laboratory Animals: Promising Results

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MA, JAK, and KA conceived and designed the study. SJ and AAA conducted experiments and analyzed samples. MA and JAK analyzed the data, while SJ and AAA drafted the manuscript. KA and MA provided critical review of the manuscript. All authors approved the final version of the manuscript for submission.

Keywords
Mastitis, Vaccine, Evaluation, Rabbits, *E. coli*, IHA

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Abstract | *Escherichia coli* is recognized as an environmental mastitis pathogen and a significant contributor to clinical mastitis cases in dairy cows. Due to environmental nature of this pathogen, conventional mastitis control measures often prove ineffective. Hence, the endeavor to combat *E. coli* mastitis through vaccination appears feasible and economically prudent. This study was aimed at preparation and evaluation of mastitis vaccines prepared by using local *E. coli* isolate, bearing the aggR gene, derived from mastitis cases in dairy cows. Prior to embarking on vaccine preparation, the immunogenicity of this *E. coli* (aggR) strain was determined across various concentrations in a cohort of 20 locally bred rabbits. For preparation of vaccines, the antigen concentration was adjusted to 10^10 cells/mL, a level determined to trigger highest IHA antibody response in rabbits. Three distinct vaccine types were prepared, each employing different adjuvants. To ensure the quality and safety of these vaccines, quality control assessment was carried out. The efficacy evaluation of the vaccines was performed on 24 locally bred rabbits, divided into 4 groups. Each group received a different type of vaccine, with booster doses at 15-day intervals following the priming. Serum samples were collected biweekly over a 60-day period, and the antibody titers were measured using IHA test. Geometric mean titers (GMT) were computed and subsequently analyzed statistically. The results indicated that the *E. coli* (aggR) isolate exhibited immunogenicity in rabbits, with the antigen concentration of 10^10 cells/mL yielding highest antibody titers. Vaccines demonstrated sterility and safety, exhibiting no adverse effects when administered to laboratory animals. Furthermore, upon comparing the effectiveness of the vaccines, it became evident that the Montanide-adjuvanted vaccine elicited significantly higher antibody titers ($P<0.05$) in comparison to the plain vaccine or the aluminum hydroxide-adjuvanted vaccine. Challenge protection assay revealed that Montanide and aluminum adjuvanted vaccines had 100% survival rate among rabbits compared to 16.7% of placebo group. It was concluded that newly developed *E. coli* (aggR) mastitis vaccines have promising results to combat *E. coli* mastitis in dairy cows.

Novelty Statement | This research presents a novel approach to combating *E. coli* mastitis in dairy cows through the development and evaluation of newly formulated vaccines. The findings offer a significant advancement in mastitis vaccine development, shedding light on potential strategies to mitigate the economic burden of mastitis in the dairy industry.

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Introduction

Mastitis, characterized by inflammation of the parenchyma of the mammary glands, poses a

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significant economic burden on dairy owners globally. It results in decreased milk production and compromises milk quality, thereby impacting the economic sustainability of dairy operations (Seegers *et al.*, 2003; Zhao and Lacasse, 2008).

Typically, mastitis is attributed to bacterial infections. These bacteria penetrate through the teat canal (Tomasinsig *et al.*, 2010), accessing the mammary gland lumen where they establish colonies. Environmental mastitis stems from bacterial pathogens present in the surroundings, while contagious mastitis results from transmission from other infected quarters (Cobirka *et al.*, 2020). These pathogens disseminate through various routes, including contaminated milk from infected cows, inadequate milking practices, bedding materials, feces, urine, and other sources of contamination (Zigo *et al.*, 2022).

Mastitis control involves several strategies, including pre and post-milking teat dipping, utilizing disposable towels for udder drying, and ensuring proper operation of milking equipment. Moreover, timely administration of antibiotics for clinical mastitis, implementing dry cow therapy, providing adequate nutrition, and upholding cleanliness and dryness in the environment are essential measures for mastitis prevention. Additionally, vaccination against mastitis is advised to prevent new infections, thereby reducing the reliance on antibiotics in livestock management (Nickerson and Ryman, 2022).

Opting for vaccination against the most prevalent pathogens presents a superior approach to mitigating mastitis-related losses. The advancement of vaccines targeting common udder pathogens has shown significant progress in recent decades (Ismail, 2017). Immunizing cows against specific pathogens responsible for intramammary infections represents a relatively novel strategy for suppressing, controlling, and preventing mastitis (Zigo *et al.*, 2021). Vaccines are now available that can reduce the duration of infection and restrict the spread of certain infectious microorganisms causing inflammation in the mammary gland across the herd (Zigo *et al.*, 2021).

Following invasion into the mammary glands, vaccinations can enhance the production of circulating antibodies against specific mastitis pathogens, thereby preventing and controlling bacterial proliferation. This heightened immunity may further diminish damage to the mammary glands caused by pathogens, facilitate tissue healing, and mitigate the clinical presentation of the disease (Nickerson and Ryman, 2022).

The most significant advancement in vaccinating cows against mastitis has come through the development of common-core gram-negative vaccines. These vaccines are formulated to target a shared element among various gram-negative pathogens. Specifically, vaccines have been formulated to combat coliform bacteria such as Escherichia, Klebsiella, and Enterobacter. This development has been prompted by the rising prevalence of mastitis caused by environmental bacteria in many cattle herds. Factors contributing to this trend include a shift towards lower somatic cell counts, heightened susceptibility of cows to coliform mastitis, and increased housing density, which elevates exposure to environmental pathogens (Nickerson and Ryman, 2022).

Furthermore, traditional herd health practices such as teat dipping and antibiotic therapy prove ineffective in managing coliform mastitis due to the persistent exposure to these pathogens in the cow's environment. The severity of coliform mastitis can vary from subclinical infections to acute clinical cases. A significant portion of clinical cases occurs within the initial three months of lactation, particularly during the first fortnight after calving, resulting in substantial losses in milk production, estimated at $100 to $300 per clinical coliform mastitis case. Hence, it is crucial for dairy farmers to either prevent the disease altogether or mitigate the risk of infection within the herd (Nickerson and Ryman, 2022). This study describes the preparation and evaluation of *Escherichia coli* (*aggR*) mastitis vaccines in laboratory animals.

Materials and Methods

Source of E. coli (aggR) *isolate*

The purified and molecularly characterized isolate of *E. coli (aggR)* was obtained from Animal Health Research Laboratory (AHRL), University of Veterinary and Animal Sciences, Lahore. The vaccinal isolates of mastitis pathogens were isolated and identified from clinical mastitis cases of dairy cattle in district Kasur under research project 'development of inactivated polyvalent vaccines for the control of mastitis in dairy cattle' funded by Punjab Agricultural Research Board (Grant No. PARB-638). The criteria for the selection of *E. coli* (aggR) type was its most common occurrence among all the *E. coli* isolates (Murrad *et al.*, 2020).

The *E. coli (aggR)* isolate was recultured on to MacConkey's agar plates and reconfirmed on the basis of colony characteristics and Gram's staining (Tripathi and Sapra, 2023). After Gram's staining further confirmation of isolate was achieved by biochemical tests and was biotyped using API 20E biochemical kit (Biomerieux, France). Molecular recharcateriztion of *E. coli* (aggR) isolate was achieved through Polymerase Chain Reaction (PCR) using forward primer 5'-GTATACACAAAAGAAGGAAGC-3 and reverse 3'- ACAGAATCGTCAGCATCAGC-5' published elsewhere (Murrad *et al.*, 2020).

Determination of immunogenicity of E. coli (aggR) *in rabbits*

Immunogenicity of *E. coli* (*aggR*) isolate was determined in rabbits. For this purpose, locally bred 20 healthy male rabbits were procured from the local market and were used for immunogenicity trials. These rabbits were kept in the laboratory animals room for 7 days before the commencement of experiments for acclimatization. Rabbits were fed on a typical rabbit diet including green fodder and fresh drinking water.

Preparation of antigen

The vaccinal isolate of *E. coli* (aggR) was cultured in brain heart infusion broth (BHI) for 24 h at 37°C. Following that, the cells were treated with formalin (0.4%, v/v) for 24 h to inactivate them. Subsequently, the inactivated cells were harvested by centrifugation at 3,000 rpm for 30 minutes at 4°C. The cells underwent two rinses with phosphate buffer saline (PBS; pH 7.2), and the resulting sediments were re-suspended in PBS. The concentrations of the antigenic preparation were then adjusted to 10^6, 10^7, 10^8, 10^9, and 10^{10} cells/mL using the spectrophotometric method at 640 nm, as outlined by Hirsch and Strauss (1964).

Inoculation of antigenic preparation in rabbits

Twenty (n=20) adult healthy male rabbits were divided randomly into 5 groups (RAA, RAB, RAC, RAD, and RAE) having 4 rabbits in each group. Rabbits in group RAA were given intraperitoneal (i.p.) antigenic reparation containing 10^6 cells/mL while group RAB was given an i.p injection of antigenic preparation containing 10^7 cells/mL. The members in group RAC was administered with antigenic preparation containing 10^8 cells/mL intraperitoneally. All rabbits in group RAD received i.p. antigenic preparation containing 10^9 cells/mL and members of group RAE were treated with antigenic preparation having 10^{10} cells/mL intraperitoneally. Rabbits in each group were given a booster shot of same antigenic preparation 2 week after priming.

Determination of antibody titer

Following the second inoculation to rabbits, serum samples were harvested from rabbits in each group weekly basis for six consecutive weeks. The antibody response was evaluated through the Indirect Haemagglutination test (IHA), following a protocol previously outlined in Rahman *et al*. (2005). The antigenic concentration that produced the highest antibody titer was chosen for vaccine formulation.

Preparation of E. coli (aggR) *mastitis vaccines*

The selected isolate of *E. coli* (*aggR*) was cultured in brain heart infusion broth for 24 h at 37°C at an orbital shaker set at 60 rpm in aerobic conditions. After confirming the purity, all suspensions were inactivated using 0.4% formalin (v/v) and then centrifuged for 1 h at 6000 rpm at 4°C and suspended in PBS. Antigenic concentration was adjusted to that which provoke the highest IHA antibody titer in rabbits. Thiomersal (0.01%) was added in as a preservative. To make the final concentration, 0.4 % formalin was added as a preservative. This was used as plain *E. coli* mastitis vaccine. To prepare Montanide adjuvanted vaccine, Montanide oil (Montanide™ ISA-W/O/W, SEPPIC, Paris France) was added to the formalin-inactivated plain vaccine and was homogenized with an equal volume of antigenic preparation drop by drop using Homogenizer (Daihan Scieitific, North America Inc., USA). Thiomersal (0.001%) and sodium azide (0.005) were added in as a preservative. For the preparation of aluminum hydroxide adjuvanted vaccine, aluminum hydroxide gel was prepared as described by (Partington, 1961). Aluminum hydroxide gel was added to plain vaccine as an adjuvant at 3.5% (Giraudo *et al*. 1997). Composition of vaccines is given in Table 1.

Table 1: Composition of *E. coli* (aggR) mastitis vaccines.

Ingredients	5 mL Dose contains		
	PEMV	MEMV	AEMV
Montanide oil (MontanideTM ISA-W/O/W, SEPPIC, Paris France)	-	3mL	-
Aluminum hydroxide 3.5%	-	-	17.5mg
Formalin (0.4%)	0.02mL	0.02mL	0.02mL
E. coli (aggR)	5×10^{10}	5×10^{10}	5×10^{10}
Thiomersal (0.001% w/v)	0.00005g	0.00005g	0.00005g
Sodium azide (0.001% w/v)	0.00005g	0.00005g	0.00005g
Phosphate buffer saline	QS	QS	QS

Preparation of placebo

Phosphate buffer saline (pH 7.2) supplemented with thiomerosal at the same concentrations as used in the vaccine was used as a placebo in the control group.

Quality control of E. coli (aggR) *mastitis vaccines*

Sterility

The sterility of each type of prepared vaccine was evaluated by streaking of loopful of vaccine onto nutrient agar plates and were incubated for 24 h at 37°C and observed for any growth.

Safety and side effects

Twenty-four (n=24) rabbits were divided into 4 groups having 6 animals in each (A, B, C, D). Each group was divided into two sub-groups having 3 rabbits in each. 0.2 mL was injected in one sub-group and 0.5 mL was injected in the other sub-group for each vaccine subcutaneously except Montanide oil adjuvanted vaccine which was injected IM. Rabbits were observed for any systemic or local reaction for 7 days.

Evaluation of E. coli (aggR) *mastitis vaccines in rabbits*

Twenty-four (n=24) adult healthy rabbits were

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selected and randomly divided into 4 groups viz., A, B, C, and D having 6 rabbits in each group. The rabbits in all groups were treated with different type of vaccine as shown in Table 2. After 15 days interval booster shot was administered to rabbits in all groups. Blood samples were collected after one week of the booster to measure antibody titers. Serum antibody titers were measured at weekly intervals for 6 weeks using IHA test.

Table 2: Experimental design for evaluation of *E. coli (aggR)* vaccines in rabbits.

Groups	Vaccine	Dose and route	Sampling days
A (n=6)	PEMV	0.2ml S/C	0, 15, 30, 45, 60
B (n=6)	MEMV	0.2ml IM	0, 15, 30, 45, 60
C (n=6)	AEMV	0.2ml S/C	0, 15, 30, 45, 60
D (n=6)	Placebo	0.2ml S/C	0, 15, 30, 45, 60

PEMV, Plain *Escherichia coli* (aggR) mastitis vaccine; MEMV, Montanide oil adjuvanted *Escherichia coli* (aggR) mastitis vaccine; AEMV, Aluminum hydroxide adjuvanted *Escherichia coli* (*aggR*) mastitis vaccine.

Challenge protection assay in rabbits

Thirty days after the booster vaccination, rabbits in each group were exposed to a 1 mL live *E. coli* (aggR) inoculum containing 10^{10} cells/mL. Mortality rates in all groups were documented until day 7 following the challenge to determine the percentage of survival.

Statistical analysis

Geometric mean titers (GMT) were calculated by taking the arithmetic mean of the log titer values and then converting it to GMT values using an antilogarithm table (Brugh, 1978). Geometric mean titer values were then analyzed statistically through repeated measures ANOVA using SAS version 19. Level of significance between groups were determined by Dunckun Mutiple Ranges (Post Hoc) test. A probability level of $P<0.05$ was considered statistically significant.

Results

Escherichia coli was gram-negative rods and gave pink color colonies on MacConkey's agar and green metallic sheen colonies on EMB agar. The isolate was citrate negative, indole and methyl red test positive and had an API 20E biochemical profile of 5154552. PCR analysis confirmed the presence of aggR toxigenic gene in *E. coli* isolate.

Evaluation of immune response at different concentrations of vaccinal isolate in rabbits

Immune response in rabbits was measured in the form of antibody titer against various concentrations of *E. coli* (aggR) by using the Indirect Haemagglutination test (IHA). It was noted from the trial that all concentrations of *E. coli* (aggR) increased antibody titer indicating an immunogenic capability of vaccinal isolate (Table 3). All of the antigenic concentrations of vaccinal isolate produced a significant immune response from day 7 (1st week), and an antibody titer peak was observed on day 28 (4th week). The antigen preparation containing 10^{10} cells/mL injected to the group RAE produced maximum antibody response as observed by the IHA antibody titer results, followed by the group RAC (10^{8} cells/mL), RAD, RAB, and RAA. The immune response of the group RAD which was injected with the dose of antigen at 10^{9} cells/mL produced less antibody titer as compared to the group RAC (10^{8} cells/mL). The maximum titer (64±0.54) in response to 10^{10} cells/mL antigen concentration was observed on day 28 (4th week) post booster and remained high till day 42 (6th week) (45.2±0.83) as compared to other groups. A similar titer was observed for all concentrations except for antigen concentration 10^{9} cells/mL where antibody titer was observed low from 4th week till the end as compared with 10^{8} cells/mL. The highest geometric mean titer was observed for the concentration 10^{10} cells/mL followed by 10^{8} cells/mL. When cumulative mean antibody titers (CMT) were compared, the antigenic concentration of 10^{10}cells/mL produced the highest CMT (45.6) followed by antigenic concentrations of 10^{8} cells/mL, 10^{9} cells/mL, 10^{7} cells/mL and 10^{6} cells/mL yielding CMT (35.9), (32.9), (30.3) and (24.6), respectively.

Evaluation of safety and side effects of vaccines

The safety and side effects of vaccines were checked after administration to rabbits. There was no mortality observed in rabbits after being administered the vaccines at 0.2mL and 0.5ml S/C doses. A moderate to severe swelling at the injection site of three rabbits was observed in the case of plain *E. coli* (aggR) mastitis vaccine. Also, an increase in rectal temperature than the normal was recorded

Table 3: Geometric mean serum IHA antibody titer values at different concentrations of *E. coli (aggR)* in rabbits

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
RAA (10^{6} cells/mL)	$6.70^{e,E} \pm 0.25$	$16.00^{d,E} \pm 0.47$	$32.00^{b,C} \pm 0.68$	$38.00^{a,D} \pm 0.60$	$32.00^{b,D} \pm 0.53$	$22.60^{c,E} \pm 0.49$
RAB (10^{7} cells/mL)	$9.50^{e,D} \pm 0.42$	$19.00^{d,D} \pm 0.58$	$38.00^{b,B} \pm 0.28$	$45.20^{a,C} \pm 0.28$	$38.00^{b,C} \pm 0.28$	$32.00^{c,C} \pm 0.70$
RAC (10^{8} cells/mL)	$13.40^{e,C} \pm 0.4$	$26.90^{d,C} \pm 0.48$	$38.10^{c,B} \pm 0.35$	$53.80^{a,B} \pm 0.47$	$45.20^{b,B} \pm 0.68$	$38.00^{c,B} \pm 0.47$
RAD (10^{9} cells/mL)	$16.00^{e,B} \pm 0.58$	$32.00^{c,B} \pm 0.46$	$39.00^{b,B} \pm 0.6$	$45.20^{a,C} \pm 0.44$	$38.00^{b,C} \pm 0.59$	$26.90^{d,D} \pm 0.40$
RAE (10^{10} cells/mL)	$19.00^{e,A} \pm 0.53$	$38.00^{d,A} \pm 0.35$	$53.80^{b,A} \pm 0.61$	$64.00^{a,A} \pm 0.54$	$53.80^{b,A} \pm 0.47$	$45.20^{c,A} \pm 0.83$

a-e superscripts on different means within the row (days) differ significantly at $p \leq 0.05$. A-E superscripts on different means within column (groups) differ significantly at $p \leq 0.05$

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Table 4: Evaluation of safety and side effects of *E. coli (aggR)* mastitis vaccines in rabbits.

Group	Vaccine	No. of rabbits	Safety of *E. coli* vaccines in rabbits				
			No. of rabbit's dose and route		Mortality	Morbidity	Symptoms
A	PEMV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	3	Moderate/Severe (+)
B	MEMV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	0	-
C	AEMV	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	1	Mild/Moderate
D	Placebo	6	3	0.2ml SC	0	0	-
			3	0.5ml SC	0	0	-

Mild/Moderate: Swelling at injection site which subsided in 48 h. Moderate/Severe: Swelling at injection site which subsided in 72-96 h. (+): Elevation of rectal temperature (40±0.5^{0}C) for 24 h post inoculation. PEMV, plain *E. coli* mastitis vaccine. For statistical abbreviations, see Table 2.

Table 5: Geometric mean IHA antibody titer values of *E. coli* (aggR) mastitis vaccines in rabbits.

Group	Vaccine	IHA antibody titers (GMT) at days				
		Day 0	Day 15	Day 30	Day 45	Day 60
A	PEMV	$1.27^{e,B}$ ± 0.14	$14.20^{d,C}$ ± 0.28	$28.50^{b,C}$ ± 0.45	$35.90^{a,C}$ ± 0.39	$22.60^{c,C}$ ± 0.27
B	MEMV	$1.80^{e,A}$ ± 0.10	$25.40^{d,B}$ ± 0.38	$57.00^{c,A}$ ± 0.29	$80.60^{a,A}$ ± 0.45	$71.80^{b,A}$ ± 0.38
C	AEMV	$2.00^{e,A}$ ± 0.14	$28.75^{d,A}$ ± 0.34	$50.80^{b,B}$ ± 0.43	$71.80^{a,B}$ ± 0.34	$35.90^{c,B}$ ± 0.34
D	placebo	$1.70^{c,A}$ ± 0.09	$1.90^{bc,D}$ ± 0.14	$2.10^{abc,D}$ ± 0.20	$2.60^{a,D}$ ± 0.21	$2.30^{ab,D}$ ± 0.19

$^{a-e}$ Superscripts on different means within row (days) differ significantly at p ≤ 0.05. $^{A-D}$ Superscripts on different means within column (groups) differ significantly at p ≤ 0.05. For statistical abbreviations, see Table 2.

when a 0.5 ml dose was administered subcutaneously, which subsided within the next 24 h and normalized. While, when administered 0.2 mL vaccine, no morbidity and mortality were observed in rabbits. The group of rabbits with MEMV showed no mortality and morbidity at any dose. Morbidity was observed in one rabbit with mild swelling at the administration site within 24 h with the dose of Aluminum hydroxide adjuvanted *E. coli* (aggR) vaccine 0.5 ml subcutaneously. Whereas, no symptoms were observed in the rabbits inoculated with 0.2 ml S/C dose of Aluminum hydroxide adjuvanted *E. coli* (aggR) vaccine. So, it was concluded from this trial that 0.2 ml of each vaccine was safe for trials in rabbits (Table 4).

Evaluation of vaccines in rabbits

Geometric mean titer (GMT) calculated by Indirect Haemagglutination Assay (IHA) revealed that the highest GMT was observed in the group of rabbits administered with Montanide oil adjuvanted *E. coli* (aggR) vaccine (MEMV) on day 30 and remained highest till day 60 as compared to other vaccine groups (Table 5). GMT values of group B started to increase on day 15 (25.4±0.38) and reached their highest on day 30 (57±0.29) as compared to other groups vaccinated with plain and aluminum hydroxide adjuvanted vaccines and reached their peak on day 45 (80.6±0.45) with a slight decrease until day 60 (71.8±0.38) followed by the rabbits in group C (vaccinated with AEMV) where the pattern was observed with the highest titer at day 45 (71.8±0.34) but a sharp decline was noted after attaining peak at day 60 (35.9±0.34). On the other hand, the A group of rabbits (vaccinated with PEMV) showed the lowest GMT compared to the two previous groups, but the same pattern was observed with maximum titer at day 45 (35.9±0.39) which declined to (22.6±0.27) at day 60. In case of rabbits of group D (unvaccinated control); baseline antibody titer was recorded lower than in other groups. Montanide oil adjuvanted *E. coli* vaccine showed higher GMT during this study followed by aluminum hydroxide adjuvanted *E. coli* mastitis vaccine (AEMV) and plain *E. coli* mastitis vaccine (PEMV). In comparison of cumulative mean antibody titers (CMT), rabbits of group B (MEMV) showed higher CMT (47.1) followed by C group (AEMV) recorded as (37.6), A group (PEMV) recorded as (20.4) and D group (unvaccinated control) (Table 6).

Table 6: Cumulative mean IHA antibody titer (CMT) of *E. coli* (*aggR*) mastitis vaccines in rabbits.

Group	Vaccine	Cumulative mean IHA antibody titers (CMT)
A	PEMV	20.4
B	MEMV	47.1
C	AEMV	37.6
D	UC	2.1

For statistical abbreviations, see Table 2.

Evaluation of vaccine challenge protection assay

None of the rabbits in the B and C groups died within 3 days after the challenge with a live inoculum of vaccine isolate containing 10^{10} cells/ml (Table 7). While only one

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rabbit from It was concluded from the study died and the survival percentage was 83.3%. In group D, only one rabbit survived which also died after 5 days. This indicated that these vaccines can be used safely in future trials. While the death of rabbits in the control group also demonstrated the lethal quality of the vaccine isolates. Therefore, the survival percentage in the vaccinated group was observed from 83 to 100% compared to the control group, where only 16.7% of the rabbits survived 7 days after the challenge.

Table 7: Survival rates in challenge protection assay of *E. coli* (*aggR*) mastitis vaccines in rabbits.

Group	Vaccine	Count of rabbits		Survival %
		Total rabbits	Count of rabbits died within 7 days	
A	PEMV	6	1	83.3
B	MEMV	6	0	100
C	AEMV	6	0	100
D	Control	6	5	16.7

For statistical abbreviations, see Table 2.

Discussion

Mastitis is a leading cause of significant economic losses in the dairy industry. This disease is caused by the interaction of the host, pathogen, and environment. Understanding the type and spread of bacteria responsible for this multi-etiological disease is critical. Among the survey's needs and benefits are estimating the actual local disease burden and identifying the major microorganisms responsible for disease production in the area. This information is required not only to develop effective treatment guidelines for existing cases, but also to develop effective control measures, because control strategies for different etiological agents differ in order to prevent new cases.

In the present study isolation and re-characterization of a selected isolate of *E. coli* (aggR) was based on standard bacteriological procedures described by (Gao *et al.*, 2017). A trial was conducted in rabbits at different concentrations (10^6, 10^7, 10^8, 10^9, and 10^{10} cell/mL) intraperitoneally to determine the immunogenicity of a purified and molecularly characterizes vaccinal isolate of *E. coli* (aggR). IHA antibody titer was measured weekly for six weeks after booster dose and found that 10^{10} cells/mL produced the best immune response in rabbits when compared to the other four concentrations. Other researchers have reported a dose-dependent humoral immune response to *E. coli*, with the highest antibody titers observed at a bacterial concentration of 10^{10} cells/mL. Additionally, it was noted that the humoral response to *E. coli* diminishes at concentrations exceeding 10^{10} cells/mL (Opdebeeck and Norcross, 1985; Watson *et al.*, 1996; Shakoor, 2006; Athar, 2007). Studies by Giraudo *et al.* (1997) and Butt (2006) have demonstrated that bacterial concentrations above 10^{10} cells/mL can lead to immunosuppression. Hence, an antigenic concentration of 10^{10} cells/mL was utilized for preparation *E. coli* (aggrR) mastitiss vaccines.

Following vaccine preparation in this study, quality control tests were conducted to assess sterility, safety, and side effects as per procedure described by Athar (2007). The findings revealed that the vaccines prepared were sterile and devoid of contamination. For safety and side effects 6 rabbits in each group with 2 subgroups were injected (PEMV= 0.2 mL SC, 0.5 mL SC; MEMV= 0.2 mL IM, 0.5 mL IM; AEMV= 0.2 mL SC, 0.5 mL SC) along with one control group. PEMV group showed moderate/severe signs at the dose of 0.5 mL SC and AEMV group showed mild/moderate side effects at 0.5 mL SC compared to MEMV group where no side effects were observed at any given dose of vaccine. It was concluded from the study that 0.2 mL did not provoke any adverse effect and was the safe dose for any *E. coli (aggR)* mastitis vaccine to check immune response in rabbits. Our findings are congruent with the findings of other workers (Giraudo *et al.*, 1997; Athar, 2007; Yousaf, 2009). In the present study rabbits vaccinated with 0.2ml and 0.5ml, Montanide oil adjuvanted vaccine did not show any local and systemic reaction due to the fact that Montanides have low viscosity and are less tissue irritant (Cook *et al.*, 1990). Our findings are in accordance with the findings of (Yousaf, 2009). In case of aluminum hydroxide adjuvanted vaccine administered at the same dose rate in rabbits resulted in a slight rise in rectal temperature and mild swelling at the injection site. Similarly demonstrated by (Butt, 2006; Athar, 2007; Ahmad and Muhammad, 2008). Our study demonstrated that the group with Montanide oil vaccine produced the highest antibody response at day 45 and then steady decline followed by aluminum hydroxide adjuvanted group where the highest antibody titer (71.80) was observed at day 45 with a similar pattern of increasing antibody titer and then started declining. For plain *E. coli* (aggR) mastitis vaccine, highest titer (35.90) was observed on day 45. When comparing all vaccines highest immune response was produced by the Montanide oil vaccine followed by aluminum hydroxide adjuvanted vaccine followed by plain *E. coli* (aggR) mastitis vaccine and the least within the control group. Similar findings were observed by Athar (2007) who reported that *E. coli* formalin-inactivated aluminum hydroxide-adsorbed polyvalent vaccine produced the highest antibody titer at day 45 followed by a non-significant decline at day 60. Then followed by *E. coli* formalin-inactivated dextran-sulphate adjuvanted polyvalent mastitis vaccine where the highest titer was observed at day 30 and gradual decrease until day 60. To determine the challenge protection essay of monovalent *E. coli (aggR)* mastitis vaccines in rabbits, twenty-four rabbits were divided into four groups with six rabbits in each group and were injected 0.2 mL vaccine SC

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and MEMV administered IM at the same dose followed by booster dose with same dose and route at day 15 after the first shot. After 30 days of the second dose, 1 mL inoculum was administered intraperitoneally. Mortality and survival rate were calculated for seven days and found that there was a 100 % survival rate in both groups with montanide oil adjuvanted and aluminum hydroxide adjuvanted vaccines. Followed by a survival rate of 83.3% in group with plain *E. coli* mastitis vaccine and the least survival rate was observed in the placebo (control) group where it was only 16.7%. Similar findings were also reported by (Giraudo *et al*. 1997; Athar, 2007; Ahmad and Muhammad 2008) where Athar (2007) found that 100% survival in vaccinated group of rabbits while 10% in placebo group.

Conclusions and Recommendations

It was concluded that the newly developed *E. coli* (aggR) mastitis vaccines have the potential to effectively combat *E. coli* mastitis in dairy cows. The study provides valuable insights into mastitis vaccine development, highlighting the importance of vaccine formulation and adjuvant selection to elicit robust immune responses and ensure protective efficacy against mastitis pathogens. Further research and field trials are needed to validate these promising results and evaluate the practical application of these vaccines on dairy farms.

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Ethical statement

All animal experiments adhered to the guidelines for the Ethical Care and Use of Laboratory Animals and received approval from the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore.

Conflict of interest

The authors have declared no conflict of interest.

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