Li *et al*. *Zoonoses* (2022) 2:40
DOI 10.15212/ZOONOSES-2022-0039

ORIGINAL ARTICLE

Development and Efficacy Evaluation of an SP01-adjuvanted Inactivated *Escherichia Coli* Mutant Vaccine Against Bovine Coliform Mastitis

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Abstract

Objective: *Escherichia coli* (*E. coli*) is one of the most common pathogens causing clinical mastitis in cattle, but no vaccine is available to prevent this disease in China. Therefore, development of an *E. coli* vaccine against bovine clinical mastitis is urgently needed.

Methods: The candidate vaccine (Ch-O111-1) and challenge (LZ06) strains were screened from milk samples of cows with clinical mastitis. To extend the cross-protection of the Ch-O111-1 strain, we deleted the galE gene fragment of the Ch-O111-1 strain through homologous recombination between the Ch-O111-1 strain and pCVD442/ΔgalE plasmid, which was identified through conventional methods, including PCR, SDS-PAGE and sequencing. The Ch-O111-1/ΔgalE (Z9) strain was characterized by extensive cross-reactivity and attenuated virulence. We prepared inactivated Z9 vaccines with different adjuvants.

Results: Immunization of inactivated Z9 antigen induced adjuvant-, dosage- and inoculation time-dependent antibody titers in cows and mice. Furthermore, immunization with SP01-adjuvanted inactivated Z9 vaccine protected cows against severe clinical mastitis caused by LZ06 and protected mice against death due to LZ06.

Conclusion: An SP01-adjuvanted inactivated Z9 vaccine was successfully developed and found to protect cows against severe mastitis caused by *Escherichia coli*.

Key words: mastitis, *Escherichia coli*, cow

Edited by:
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Two reviewers chose to be anonymous

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Received: October 10 2022
Revised: November 10 2022
Accepted: November 15 2022
Published Online: December 09 2022

INTRODUCTION

Bovine mastitis is one of the most severe diseases in the dairy industry and causes serious economic losses in the milk industry worldwide [1–4]. The morbidity and mortality of lactating cows are commonly associated with bacterial infections [2,5–9]. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Streptococcus* are the three main bacteria causing bovine mastitis [1]. Among them, *E. coli* is considered the main causative agent of clinical-type mastitis in cows, and it is widely present in the environment and can invade the udder tissues of cows through various pathways, thereby triggering an inflammatory response that leads to bovine mastitis [5,9].

Although antibiotics are an optimal treatment for bovine clinical mastitis

[10-13], the massive use of antimicrobial drugs has led to the emergence of drug-resistant strains of *E. coli*, thus making effective prevention and treatment of mastitis extremely difficult [9,14-18]. In the context of the current advocacy of resistance reduction initiatives, methods using biological agents against mastitis are being developed, including phage therapy, antimicrobial therapy, antibody therapy, nonspecific immune boosting prophylaxis and vaccine prophylaxis. In particular, to effectively prevent and control the occurrence of bovine mastitis and to prevent the further effects of antibiotics on the ecosystem, the development of new vaccines has become a critical research direction [19-23]. At present, bovine mastitis *E. coli* vaccine is available from the U.S. company Schotten and from the Spanish company Ophthalmology. Although it had been used in the 2000s in China, the J5 vaccine disappeared from the market shortly thereafter because its protective efficacy against bovine mastitis did not meet farmers' expectations [24-28]. No vaccine is currently available to prevent bovine mastitis in China. Therefore, safer and more effective vaccines against mastitis must be developed in the post-antibiotic era.

To develop a safe and effective vaccine against bovine clinical mastitis primarily attributed to *E. coli* infection, we isolated, reconstructed and screened the candidate vaccine strain through conventional methods. Furthermore, the protective efficacy of the prepared vaccine was evaluated in established animal models in this study.

MATERIALS AND METHODS

Adjuvants, media and reagents

$Al(OH)_3$ and oil emulsion adjuvant (Duoprime PenReco) were purchased from Brenntag Biosector, Denmark and Calumet, USA, respectively. The SP01 adjuvant was prepared by Beijing Institute of Microbiology and Epidemiology as previously described [29,30]. Lysogeny broth (LB), lysogeny agar (LA), tryptic soy broth (TSB), tryptic soy agar (TSA) and MacConkey agar (MCA) were purchased from BD, USA. *E. coli* serotype determination kits were purchased from Japan Biology Institute and Denmark Statens Serum Institute Co., Ltd, Japan.

Animals

Balb/c mice were provided and housed under standard laboratory conditions by the Laboratory Animal Center, Academy of Military Medical Science. Three-year-old Holstein cows were provided and housed in clean separation rooms by Inner Mongolia Chifeng Boen Pharmaceutics Co, Ltd. All animal protocols were reviewed and approved by the Academic Animal Care and Use Committee (No. IACUC of AMMS-2020-030).

Milk and serum sample collection

The udders of the cows were washed with warm water and sterilized with 0.2% benzalkonium bromide and 75% alcohol; subsequently, 5 ml milk per udder was collected. Blood samples were collected, kept at room temperature for 3 hours and centrifuged at 1200×g for 10 minutes. Then the sera were collected and stored at −20°C for ELISA.

Isolation of clinical *E. coli* strains from milk samples of cows with clinical mastitis

Milk samples were spread on microscopic slides and subjected to Gram staining, and then milk samples with Gram-negative bacteria were then streaked on MCA and incubated at 37°C for 16 hours to observe the bacterial morphology. The milk sample cultures showing *E. coli* shape and color were streaked and inoculated on TSA at 37°C for 16 hours. Typical bacteria colony was cultured in TSB at 37°C for 16 hours, stored at −80°C, and further analyzed.

E. coli serotype identification

Serotypes of *E. coli* isolates were determined by slide agglutination with sera of the major serotypes of *E. coli* causing bovine coliform mastitis reported in the current study. Briefly, the bacterial suspension was mixed with the indicated anti-*E. coli* O antigen antibody (e.g., O2, O8, O21, O81, O86 or O111) for 15 minutes and observed.

Screening of candidate *E. coli* vaccine or challenge strains

The purity, biochemistry and virulence of *E. coli* strains were determined with conventional methods. The primary typical *E. coli* strain was screened for further vaccine studies. The virulent *E. coli* strain was chosen for the establishment of animal models (detailed methods in Supplementary file 1).

Construction of the Ch-O111-1 mutant strain (Ch-O111-1/ΔgalE)

To further extend the serological cross-reactivity with a variety of Gram-negative bacteria, we deleted the galE gene of the Ch-O111-1 strain through homologous recombination according to previously described methods [31], as shown in Fig 1. Briefly, the recombinant suicide plasmid pCVD442/ΔgalE was constructed by amplification of the up- and downstream fragments by PCR with the indicated amplification primers in S1 Table, and cloning into the pCVD442 plasmid through restriction enzyme digestion (Sac I and Xba I) and ligation (T4 DNA ligase). The pCVD442/ΔgalE plasmid was translated into DH5a competent cells, amplified and further electrotransformed into SM10 competent cells. The SM10 (pCVD442/ΔgalE) and Ch-O111-1 (NalR) strains were cultivated in LB at 37°C until the OD_{600} was approximately 0.8, mixed and filtered onto nylon membranes. The filter membrane was cultured at 37°C overnight on LA without antibiotics. Ch-O111-1 (pCVD442/ΔgalE) was obtained through anti-nalidixic acid (Nal 50 μg/ml) and anti-ampicillin (Amp 100 μg/ml) screening of the 0.01 mol/L $MgSO_4$ washed bacteria and was designated Ch-O111-1/ΔgalE.

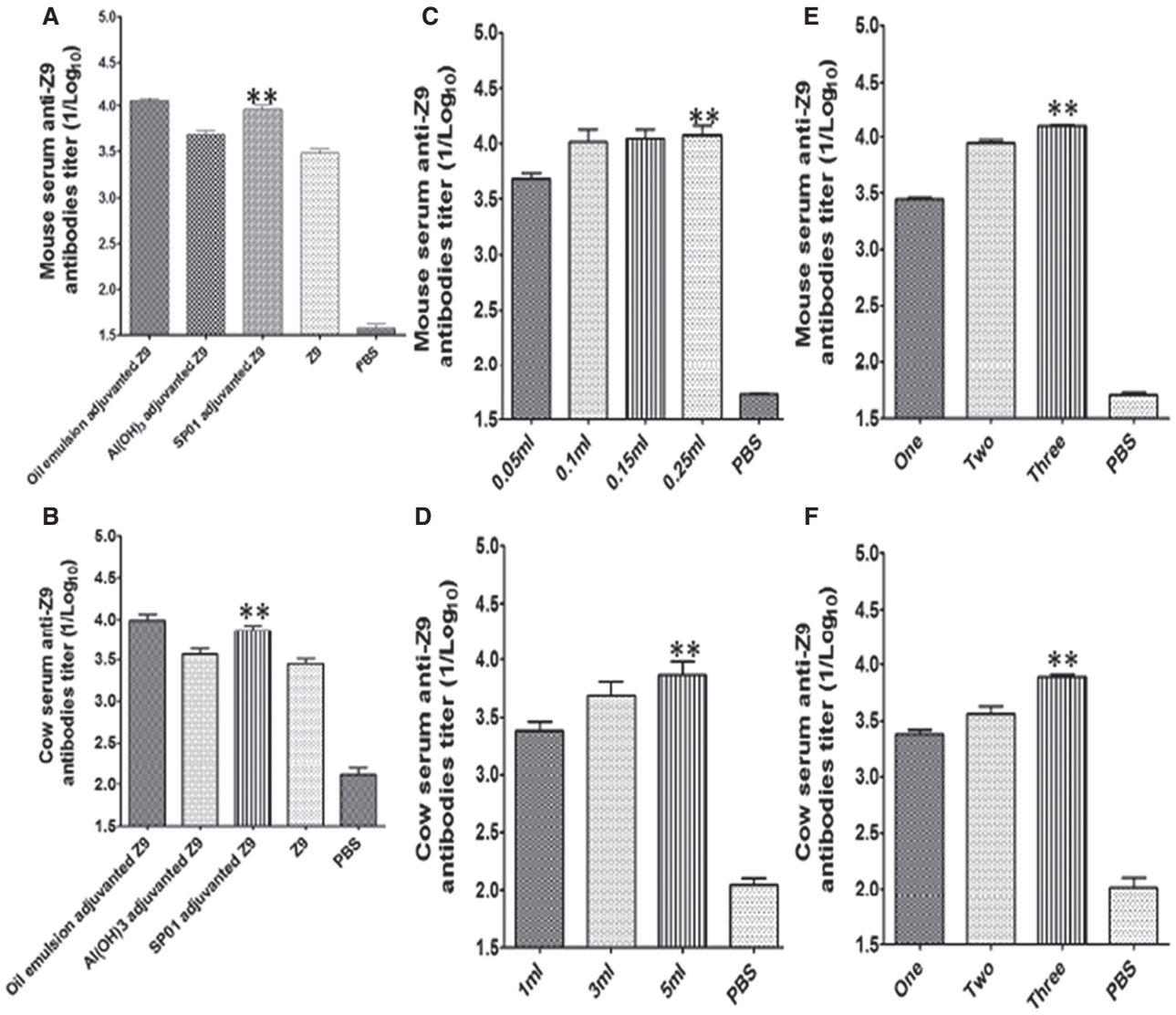

FIGURE 1 | Immunization of *E. coli* CH-O111-1/ΔgalE (Z9) antigens induced adjuvant-, dosage-, times-dependent anti-Z9 antibodies in mice and cows. (A) Six-week-old Balb/c mice (n=10 per group) were inoculated s.c. with the indicated preparations (0.25×10^{10} CFU of inactivated Z9 in 0.25 ml) once every 14 days for 3 times, and the sera were collected and determined by ELISA 14 days post the third inoculation. (B) Three-year-old Holstein cows (n=5 per group) were inoculated s.c. with the indicated preparations (5×10^{10} CFU of inactivated Z9 in 5 ml) once every 28 days for 3 times, and the sera were collected and determined by ELISA 28 days post the third inoculation.**, $P<0.01$, SP01-adjuvanted inactivated Z9 preparation compared with inactivated Z9. (C) Six-week-old Balb/c mice (n=10 per group) were inoculated s.c. with the indicated dosages (1×10^{10} CFU of inactivated Z9 per ml) once every 14 days for 3 times, and the sera were collected and determined by ELISA 14 days post the third inoculation. **, $P<0.01$, 0.05 ml compared with 0.25 ml. (D) Three-year-old Holstein cows (n=5 per group) were inoculated s.c. with the indicated dosages (1×10^{10} CFU of inactivated Z9 per ml) once every 28 days for 3 times, and the sera were collected and determined by ELISA 28 days post the third inoculation.**, $P<0.01$, 5 ml compared with 1 ml. (E) Six-week-old Balb/c mice (n=10 per group) were inoculated s.c. with 0.25×10^{10} CFU of SP01-adjuvanted inactivated Z9 once every 14 days for 3 times, and the sera were collected and determined by ELISA 14 days post inoculation. (F) Three-year-old Holstein cows (n=5 per group) were inoculated s.c. with 5×10^{10} CFU of SP01-adjuvanted inactivated Z9 once every 28 days for 3 times, and the sera were collected and determined by ELISA 28 days post inoculation.**, $P<0.01$, three times compared with one.

Identification of the strain Ch-O111-1/ΔgalE (Z9)

The galE gene fragments of Ch-O111-1 and Ch-O111-1/ΔgalE (Z9) were determined by PCR and sequencing with the indicated identification primers in S1 Table. The lipopolysaccharide (LPS) of Ch-O111-1 and Ch-O111-1/ΔgalE (Z9) was extracted with an LPS Extraction Kit, separated by SDS-PAGE and stained with a Silver Stain Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's specifications. The bovine anti-Z9 serological cross-reactivity with

a variety of clinical *E. coli* strains was detected through ELISA.

Determination of virulence in mice and cows

The virulence of each candidate *E. coli* strain was determined in 12-week-old Balb/c mice. The virulence of the mouse-lethal *E. coli* strain was further assayed in 3-year-old lactating Holstein cows by determination of the rectal temperature, electrical conductivity of milk (ECM, with a hand-held mastitis detection instrument, type DRAMINSKI MA-1, Beijing Fubangshengye Science and Technology Co, Ltd) and bacterial concentration through conventional methods. To establish the mouse and cow mastitis models, we performed detailed assessment of the virulence of the *E. coli* LZ06 strain details in mice and cows. Briefly, LD_{50} was determined by intraperitoneal (i.p.) injection of 3.8×10^6, 3.8×10^7 or 3.8×10^8 CFU of LZ06 per mouse into 12-week-old Balb/c mice (ten mice per group). The virulence of LZ06 was further assayed by intramammary (i.m.) inoculation of 500, 1000 or 2000 CFU per cow into 3-year-old lactating Holstein cows (five cows per group).

Preparation of various Z9 vaccines

The Z9 strain was cultured in TSB at 37°C until the OD600 was approximately 20, inactivated by 0.4% formaldehyde at 37°C for 60 hours, washed and suspended in PBS, and stored at 4°C for 30 days. $Al(OH)_3$-adjuvanted inactivated Z9 vaccine was prepared through conventional methods, and the final $Al(OH)_3$ and Z9 concentrations were 1.2 mg/ml and 1×10^{10} CFU/ml, respectively. Oil emulsion-adjuvanted inactivated Z9 vaccine was prepared through conventional methods, and the final Z9 concentration was 1×10^{10} CFU/ml. SP01-adjuvanted inactivated Z9 vaccine was prepared through previously described methods [29,30], and the final Z9 concentration was 1×10^{10} CFU/ml.

Mouse and cow protection assays

Six-week-old Balb/c mice (ten mice per group) were subjected to subcutaneous (s.c.) inoculation with the indicated vaccines once every 14 days three times. Then the immunized mice were challenged by i.p. injection of 3.8×10^8 CFU per mouse 14 days after the third immunization, and the survival rates were observed. The sera were collected at the indicated times and determined with ELISA.

Three-year-old lactating Holstein cows (five cows per group) were subjected to s.c. inoculation with the indicated vaccines once every 28 days three times. Then the immunized cows were challenged by i.m. injection of 1000 CFU per cow 28 days after the third immunization; the clinical symptoms were observed, and the rectal temperature was determined. The sera were collected at the indicated times and analyzed with ELISA. The milk was collected at the indicated times and used for determining ECM and bacteria concentrations.

ELISA

The presence of serum IgG specific to Z9 or the indicated clinical isolates was determined by indirect ELISA. Briefly, 0.1 ml of 1×10^9 CFU/ml inactivated Z9 or the indicated clinical isolates was used to coat the wells. The cutoff value for the serum IgG assay was calculated as the mean specific OD plus standard deviation (SD) for 20 serum samples assayed at a dilution of 1:25 from the unimmunized animals. The serum titer was calculated as the reciprocal of the highest serum dilution yielding a specific OD higher than 2.1× the cutoff value. The absorbance of the developed color was measured at 450 nm (OD450). All assays were performed in triplicate and repeated three times.

Statistics

All quantitative data are expressed as the mean ± SD, performed by one way ANOVA following Student's *t*-test in GraphPad Prism (version 5.0, GraphPad Software, CA). Survival curves were analyzed with log rank tests. A P value < 0.05 was considered significant.

RESULTS

The candidate vaccine or challenge strain for developing bovine coliform mastitis vaccine was obtained from many milk samples from cows with clinical mastitis

The history of the candidate bovine mastitis vaccine strain is shown in S1 Fig. Briefly, 344 coliform strains were isolated from 1321 milk samples, which were collected from cows with clinical mastitis from many dairy farms in different regions of China. The serotype identification results indicated that the ratios of O111, O2, O8, O21, O81, O86 and other strains in the isolated *E. coli* strains were 47.1%, 11%, 12.2%, 6.7%, 10.5%, 6.9% and 5.5%, respectively. We randomly chose seven typical O111 strains for further identification. Through conventional methods according to Bergey's Manual of Determinative Bacteriology, we assessed their biological characteristics, including Gram-staining, MCA culture, biochemical test and purity identification, and found results consistent with *E. coli* (data not shown). Furthermore, we assayed the cross-reactivities of cow anti-Ch-O111-(1–7) antibodies with the isolated clinical strains with ELISA. The results (Table 1) indicated that Ch-O111-1 had good cross-reactivity above 40%, which was higher than that of the other strains (data not shown). Thereafter, we chose the Ch-O111-1 strain for developing the bovine coliform mastitis vaccine.

To obtain the candidate challenge strain for evaluating the protective efficacy of the vaccines against bovine coliform mastitis, we determined the LD_{50} values of nine typical *E. coli* strains in 12-week-old Balb/c mice, which were between 1×10^6 and 1×10^8 CFU. Three mouse-lethal *E. coli* strains were further assayed in 3-year-old lactating Holstein cows. The *E. coli* LZ06 strain induced clinical mastitis in the infected cows, whereas the others did not (S1 Fig). We further assayed the virulence of LZ06 in

TABLE 1 | Reactivity ratios of anti-Ch-O111-1 antibodies with *E. coli* clinical isolates.

	Serotype					Total
	O2	O8	O21	O81	O86	
Experimental strains	12	15	9	14	10	60
Positive strains	6	6	5	9	4	30
Ratio (%)	50.00	40.00	55.56	64.29	40.00	50.00

The wells were coated with the indicated 1×10^8 CFU/ml inactivated clinical bacteria isolates as described in the text. The reactivity of cow anti-Ch-O111-1 antibodies was determined with ELISA. The clinical isolate's OD420nm value was more than negative control (healthy Holstein cow serum) OD420nm mean value was designated reactivity positive.

the mice and cows in detail. The data in S2 Fig showed that the LD_{50} of LZ06 was 3.8×10^7 CFU. Furthermore, more than 1000 CFU of LZ06 induced marked clinical mastitis in 3-year-old lactating Holstein cows, which was characterized by high rectal temperature, high bacterial concentrations and low ECM. Thus, we established the mouse model by i.p. infection of 3.8×10^8 CFU LZ06 in 12-week-old Balb/c mice and in the bovine mastitis model by i.m. inoculation of 1000 CFU LZ06 in 3-year-old lactating Holstein cows.

Knockout of the galE gene of Ch-O111-1 was characterized by extensive cross-reactivity and attenuated virulence

As shown in S3 Fig, the Ch-O111-1 strain without the galE gene was obtained by recombination between the pCVD-galE-u/d plasmid and wild type Ch-O111-1 strain. pCVD-galE-u/d was constructed through enzyme digestion and ligation between pCVD442 and pMD18-T-galE-u/d. The pMD18-T-galE-u/d plasmid was constructed through ligation between the pMD18-T plasmid and 250 bp up- and downstream fragments of the galE gene. The 250 bp up- and downstream fragments of the galE gene were amplified by PCR with the primers shown in Table 2 and analyzed by electrophoresis. The correct pMD18-T-galE-u/d and pCVD-galE-u/d plasmids were all screened and identified through restriction enzyme (Sac I and Xba I) digestion and electrophoresis and yielded results consistent with the plasmid design.

TABLE 2 | Primers for amplification of the up- and downstream fragments of galE.

Primer name	Primer sequence, 5′-3′
galE-u-F(F1)	TCGGACGGTGGGCTCTATCGCTATG
galE-u-R(R1)	TGTAGGCCGGAGAGGGGGCTTACGC
galE-d-F(F2)	GGGCGGACGCCAGCAAAGCCGACCGTGAA
galE-d-R(R2)	GCAACGCCATCAAAGGATCGTTGCT

After anti-nalidixic acid and anti-ampicillin screening, two of nine randomly chosen strains were Ch-O111-1 without the galE gene (Fig 2). Compared with the 1634 bp PCR products in strains 1–7, only 524 bp fragments were observed in strains 8 and 9, thus suggesting that recombination occurred in strains 8 and 9 (Fig 2A). The silver-stained LPS extraction results indicated that the LPS of strains 8 or 9 was incomplete, thus further verifying the function of the galE gene in LPS synthesis (Fig 2B). We randomly chose strain 9 for galE gene sequencing. The data in Fig 2C showed that, compared with the wild type Ch-O111-1 strain, the underlined 1110 bp galE gene fragment of strain 9 shown in Fig 2 was deleted, and the strain was designated Ch-O111-1/ΔgalE (Z9).

To further explore the biological characteristics of Ch-O111-1/ΔgalE (Z9), we determined its virulence in 12-week-old Balb/c mice and the cross-reactivity of cow anti-Z9 sera with the isolated clinical strains. The LD_{50} of Z9 for 12-week-old Balb/c mice was 2×10^8 CFU, and that of the maternal strain Ch-O111-1 was 1.8×10^7 CFU, thus suggesting that the virulence of Z9 was attenuated. As shown in Tables 1 and 3, compared with the maternal strain Ch-O111-1, the mutant strain Z9 showed a markedly extended cross-reactivity ratio from approximately 50% to more than 90%, thus suggesting that Z9 might have conferred extensive cross-protection.

Immunization with different Ch-O111-1/ΔgalE (Z9) antigens induced multiple anti-Z9 antibody levels in mice and cows

To further validate the safety of Z9 for cows and the environment, we chose the inactivated Z9 as an antigen. For inactivated antigens, the choice of a suitable adjuvant is important to elicit optimal protection against infection. The data in Fig 1A and 1B indicated that, compared with the simple inactivated Z9, all adjuvanted Z9 induced higher levels of anti-Z9 antibodies. However, oil emulsion adjuvanted Z9 caused severe local inflammation (data not shown). Furthermore, SP01-adjuvanted Z9 induced higher levels of anti-Z9 antibodies than $Al(OH)_3$-adjuvanted Z9. Therefore, we chose SP01 as the adjuvant for the development of the bovine coliform mastitis vaccine. The immunization regime is also a key factor enhancing the protective efficacy of the vaccines against infection. As shown in Fig 1C and 1D, the anti-Z9 antibody titers increased when the vaccine dose was increased to 0.25 ml in mice and to 5 ml in cows. The data in Fig 1E and 1F showed that the anti-Z9 antibody titers increased when the number of inoculation times was increased to three.

Therefore, 0.25 ml of the SP01-adjuvanted inactivated Z9 vaccine was used for s.c. inoculation in mice once every 14 days three times, and 5 ml in cows once every 28 days three times, to evaluate the protective efficacy against *E. coli* caused diseases.

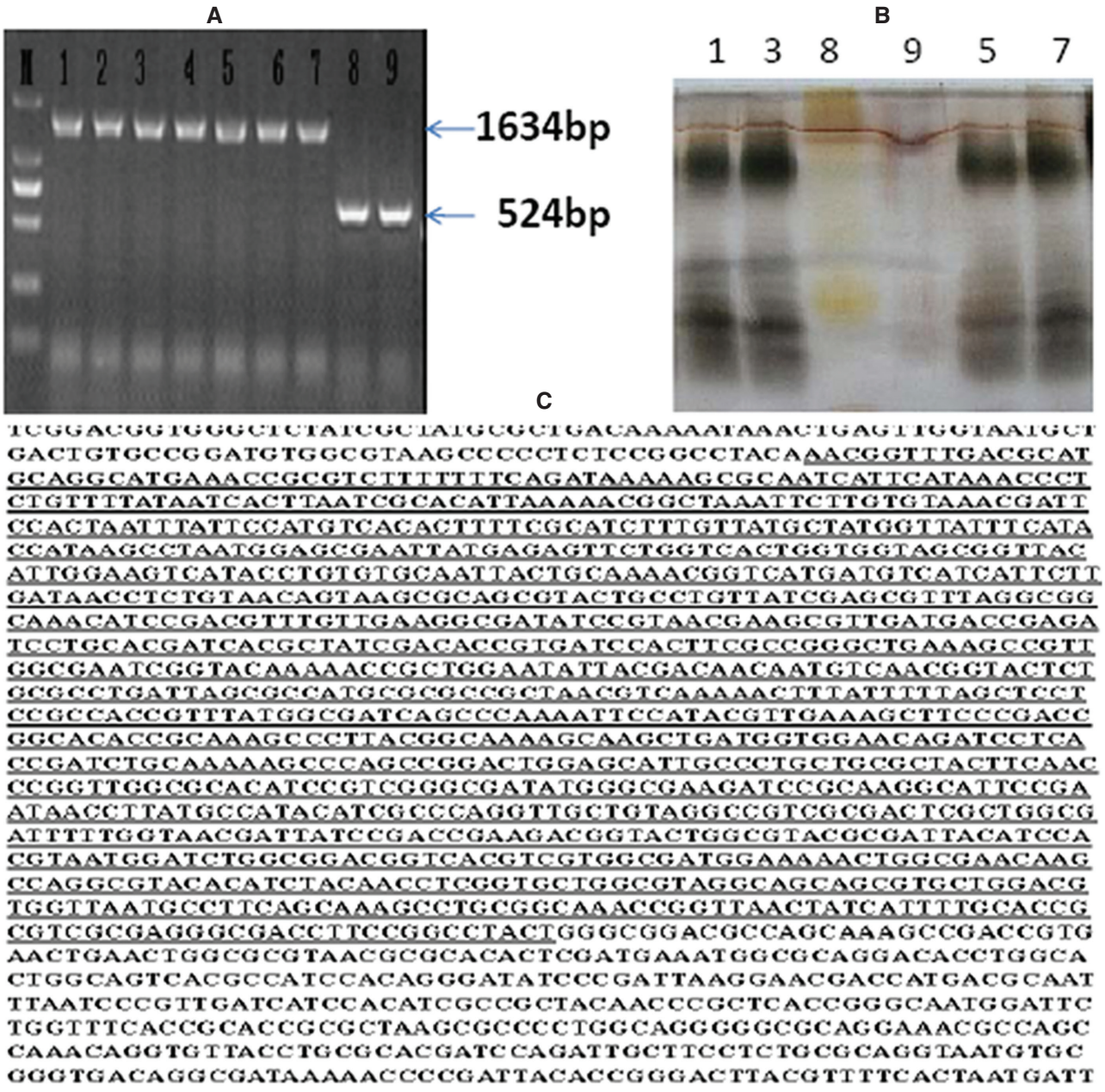

FIGURE 2 | *E. coli* CH-O111-1/ΔgalE (Z9) strain verification by PCR, SDS-PAGE and sequencing. (A) Homologous recombination between the pCVD-galE-u/d and wild type CH-O111-1 strain, screened by PCR. Strains 8 and 9 were *E. coli* CH-O111-1/ΔgalE strains, and strains 1–7 were wild type strains in which homologous recombination did not occur. (B) LPS of strains 1, 3, 5, 7, 8, and 9, separated by SDS-PAGE and stained with a fast silver stain kit. LPS was absent in strains 8 and 9. (C) The GalE fragment of strain 9 was sequenced, and the underlined part was deleted.

Immunization with the SP01-adjuvanted inactivated Z9 vaccine protected cows against severe clinical mastitis caused by LZ06 and protected mice against death due to LZ06

As shown in Fig 3, immunization with the SP01-adjuvanted inactivated Z9 vaccines protected mice and cows against LZ06 caused diseases. Compared with SP01 inoculation, inoculation with the SP01-adjuvanted inactivated Z9 vaccine markedly protected mice against death due to LZ06, with a protection efficacy of approximately 100% (Fig 3A). Furthermore, immunization with SP01-adjuvanted inactivated Z9 vaccine ameliorated clinical symptoms in the LZ06 challenged cows. In contrast to the SP01 inoculated group, in which the rectal temperatures (Fig 3B), ECM (Fig 3C) and the live bacterial concentrations (Fig 3D) in challenged cows were all abnormal, LZ06-challenged cows immunized with the SP01-adjuvanted inactivated Z9 vaccine were protected against severe mastitis.

TABLE 3 | Reactivity ratios of anti-Z9 antibodies with *E. coli* clinical isolates.

	Serotype					Total
	O2	O8	O21	O81	O86	
Experimental strains	12	15	9	14	10	60
Positive strains	11	14	9	13	9	56
Ratio (%)	91.67	93.33	100	92.86	90.00	93.33

The wells were coated with the indicated 1×10^8 CFU/ml inactivated clinical bacteria isolates as described in the text. The reactivity of cow anti-Z9 antibodies was determined with ELISA. The clinical isolate's OD420nm value was more than negative control (healthy Holstein cow serum) OD420nm mean value was designated reactivity positive.

DISCUSSION AND CONCLUSION

In this study, an SP01-adjuvanted inactivated Z9 vaccine was developed, which prevented cows from developing severe clinical mastitis. The Z9 vaccine strain was obtained by screening the isolates and deleting the galE gene fragment of the wild type strain, which was characterized by extensive cross-reactivity and diminished virulence. The inoculation regime of the SP01-adjuvanted inactivated Z9 vaccine was formulated by optimizing the adjuvant, dosage and inoculation times, and was evaluated in established animal models with the virulent clinical isolate LZ06 in pre-clinical studies. Clinical trials of the SP01-adjuvanted inactivated Z9 vaccine were performed according to the guidelines of the Agriculture Administration of China (No. 2014081).

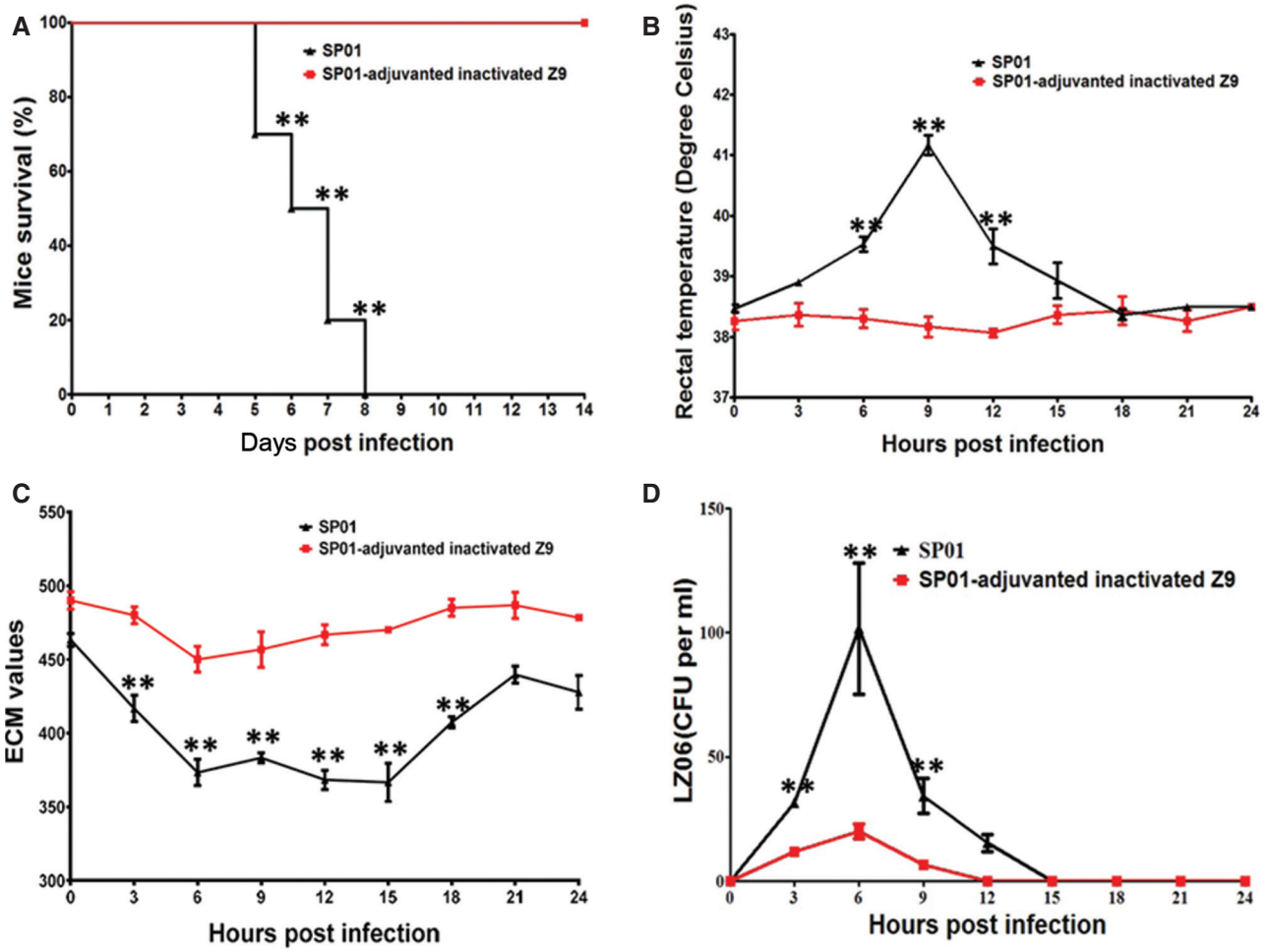

FIGURE 3 | Immunization with SP01-adjuvanted inactivated Z9 vaccine protected mice and cows against diseases caused by LZ06. (A) Inoculation with SP01-adjuvanted inactivated Z9 vaccines protected mice against death due to LZ06. Six-week-old Balb/c mice (n=10 per group) were subjected to s.c. inoculation with the vaccine or SP01 (0.25 ml per mouse) once every 14 days three times, and i.p. infection with 3.8×10^8 CFU of LZ06 14 days after the third infection. Mortality was monitored and recorded daily after infection for 14 days. (B, C, D) Inoculation with SP01-adjuvanted inactivated Z9 vaccine ameliorated clinical symptoms in LZ06 challenged cows. Three-year-old Holstein cows (n=5 per group) were subjected to s.c. inoculation with the vaccines or SP01 (5 ml per cow) once every 28 days three times, and i.m. infection with 2000 CFU of LZ06 28 days after the third infection. Rectal temperature (B), ECM value (C) and concentrations of live bacteria (D) were determined and recorded every 3 hours after infection for 24 hours. Representative results of three independent experiments are shown. **, $P<0.01$, SP01 compared with the vaccine.

E. coli is a primary pathogen causing bovine clinical mastitis, according to our studies and worldwide reports [20,32-34]. Commercial J5 bacterins have been used in many countries for approximately 30 years, but many controversies exist regarding their protective efficacy against bovine mastitis [24-26,28,35-44], which may be attributable to the vaccine strain J5 not always providing adequate protection in diverse countries with different prevalent *E. coli* strains [9,34,45,46]. In China, the commercial J5 vaccine was used in the 2000s and disappeared from the market soon thereafter because its efficacy against bovine mastitis was often low in clinical use. Therefore, we screened candidate vaccine strains by investigating local clinical isolates. The O111 antigen-group of *E. coli* was prevalent in China, according to the serotype identification, accounting for 47.1% of the clinical isolates. Therefore, we chose the O111 antigen-group of *E. coli* for further vaccine strain screening.

Seven typical O111 strains were designated Ch-O111-(1–7), and their cross-reactivity was analyzed with ELISA. To avoid false positives, we designated clinical isolates with a mean optical density more than negative control OD420nm mean value as having positive reactivity. Among these seven strains, Ch-O111-1 had the most extensive cross-reactivity, with positivity ratios between 40% and 65%, thus further suggesting that the J5 vaccine might have low cross-reactivity with isolates in China. Although Ch-O111-1 had relatively high cross-reactivity, it could not satisfy the clinical need for a broad and effective mastitis vaccine against diverse *E. coli* if the prevalent strains in the specific farms were to lack high cross-reactivity with Ch-O111-1. Therefore, we chose the Ch-O111-1 strain for further reconstruction.

LPS, the outer layer of the outer cell wall in the Gram-negative bacteria, comprises a variable oligosaccharide region associated with a conserved core polysaccharide and lipid A regions. The variable oligosaccharide region determines the bacterial serotype. However, the core polysaccharides are highly conserved and shared by the major species, genera and groups of Gram-negative bacteria. According to the level of mutation in the outer layer, R-mutants are grouped into a, b, c, d and e [38,42,45,47-52]. The J5 strain is an Rc-mutant without uridine diphosphate galactose epimerase (galE), which lacks linkage between galactose and glucose in the core antigen [42]. The 'O' exposure mutant may be a suitable antigen strain that can provide protective immunity against a wide range of Gram-negative bacterial infections [45,52]. To extend the cross-reactivity, we deleted the galE gene of the Ch-O111-1 strain through homologous recombination. Many tools have been described for producing mutants in bacterial chromosomes [31,51,53,54]. In this study, the suicide plasmid pCVD442, which carried R6K ori, the replication origin of plasmid R6K, the mob and bla regions of the suicide vector pGP704, the sacB gene of B. subtilis and five unique restriction endonuclease sites, was used for reconstructing the galE gene deleted mutant of the Ch-O111-1 strain [31,51]. The Ch-O111-1 strain without the galE gene was constructed and designated Ch-O111-1/ΔgalE, and was characterized by diminished virulence and extensive cross-reactivity (more than 90%) with many serotypes of *E. coli*. Therefore, the Ch-O111-1/ΔgalE strain was used for the development of the bovine mastitis vaccine.

The magnitude, duration and effectiveness of mammary gland immunity depends on the prepared antigens used in vaccination [21,22,40,43]. Although $Al(OH)_3$ and oil emulsion adjuvants have been broadly used in inactivated veterinary vaccines, the SP01 adjuvant has many advantages in safety and immunity enhancement [20,29,30]. The immunization regime of the SP01-adjuvanted inactivated Z9 vaccine, including the dosage and inoculation times, was in accordance with that of the J5 vaccine [26,36,39,44].

To evaluate the protective efficacy of the SP01-adjuvanted inactivated Z9 vaccine against bovine mastitis, we established suitable animal models with the isolate LZ06 in 12-week-old mice and 3-year-old Holstein cows. Although the mouse-lethal model was not suitable for evaluating the protective efficacy of the vaccine against bovine mastitis, it provided an inexpensive reference for the quality of the vaccine in the pre-clinical studies. Our findings in the established Holstein cow model were consistent with previously reported data [36,37] indicating abnormal rectal temperatures and bacterial concentrations. Although infection with more CFUs of bacteria results in more severe clinical mastitis in cows, the animal ethics and bio-safety did not permit us to test exposure at those levels. In these established animal models, the SP01-adjuvanted inactivated Z9 vaccine markedly prevented severe diseases in infected animals.

In conclusion, the SP01-adjuvanted inactivated Z9 vaccine was successfully developed and demonstrated to protect cows against bovine clinical mastitis caused by *E. coli*.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (2019SWAQ05-3-5 and 2019YFC1200604), Inner Mongolia Science and Technology guide projects (2018-high-significance-4, 201702097) and "863" projects (2011AA10A210).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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