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Development and efficacy evaluation of a 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, and there is no available vaccine to prevent this disease in China; therefore, there is an urgent need to develop an *E. coli* vaccine against bovine clinical mastitis. Methods: the candidate vaccine (Ch-O111-1) and challenge (LZ06) strains were screened from the milk samples of the cows with clinical mastitis. Furthermore, to extend the cross-protection of the Ch-O111-1 strain, the galE gene fragment of the Ch-O111-1 strain was deleted by homologous recombination between the Ch-O111-1 strain and pCVD442/△galE plasmid, which was identified by the conventional methods, including PCR, SDS-PAGE and sequencing. The Ch-O111-1/ΔgalE (Z9) strain was characterized by extensive cross-reactivity and attenuated virulence, and we prepared inactivated Z9 vaccines with different adjuvants. Results: Immunization of inactivated Z9 antigen induced adjuvant-, dosage-, inoculation times-dependent antibody titers in cows and mice. Furthermore, immunization with SP01-adjuvanted inactivated Z9 vaccine protected cows from LZ06 caused severe clinical mastitis and mice from LZ06 caused death. Conclusion: SP01-adjuvanted inactivated Z9 vaccine was successfully developed, which could protect the cows from *Escherichia coli* caused severe mastitis.

Key words: Mastitis; *Escherichia coli*; Cow

1. Introduction

Bovine mastitis is one of the most severe diseases in the dairy and causes serious economic losses to milk industry worldwide[1-4]. Morbidity and mortality of the

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lactational 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 to be the main causative agent of clinical-type mastitis in cows and is widely present in the external environment and can invade the udder tissues of cows through various pathways, triggering an inflammatory response that leads to bovine mastitis [5,9].

Although antibiotics are one of the optimal treatment regimes for bovine clinical mastitis currently[10-13], the massive use of antimicrobial drugs has led to the emergence of drug-resistant strains of *E. coli*, making it extremely difficult to effectively prevent and treat mastitis[9, 14-18]. In the context of the current advocacy of resistance reduction initiatives biologics against mastitis are on the agenda, including phage therapy, antimicrobial therapy, antibody therapy, non specific immune boosting prophylaxis and vaccine prophylaxis. In particular, in order to effectively prevent and control the occurrence of bovine mastitis and to prevent the further impact of antibiotics on the ecosystem, the development of new vaccines has become the most important direction to explore[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 2000's in China, the J5 vaccine disappeared soon in the markets because its protective efficacy against bovine mastitis did not reach the farmer's anticipations[24-28]. There is no available vaccine to prevent bovine mastitis in China now. It is urgent that the more safe and effective vaccines against mastitis are developed in the post-antibiotics era.

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

2. Materials and methods

2.1. Adjuvants, mediums and reagents

$Al(OH)_3$ and Oil emulsion adjuvant (Duoprime PenReco) were purchased from Brenntag Biosector, Denmark and Calumet, USA, respectively. SP01 adjuvant was prepared by Beijing Institute of Microbiology and Epidemiology as described before[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. The *Escherichia coli* serotype determination kits were purchased from Japan Biology Institute and Denmark Statens Serum Institute Co., LTD, Japan.

2.2. Animals

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

2.3. Milk and serum samples collection

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

2.4. Isolation of clinical *Escherichia coli* strains from the milk samples of the clinical mastitis cows

Milk samples were spread on microscopic slides with Gram staining, and then the milk samples with Gram-negative bacteria were streaked on MCA at 37°C for 16 hours to observe the bacteria morphology. The milk samples with *Escherichia coli* shape and color were streaked and inoculated on TSA at 37°C for 16 hours. The typical bacteria colony was cultured in TSB at 37°C for 16 hours, preserved at -80°C, and determined further.

2.5. Escherichia coli serotype identification

Serotypes of *Escherichia coli* isolates were determined by slide agglutination according to the provided specifications. Briefly, the bacteria suspension was mixed with the indicated anti-*Escherichia coli* O antigen antibody (O2, O8, O21, O81, O86, or O111, etc.) for 15 minutes and observed.

2.6. Screening the candidate *Escherichia coli* vaccine or challenge strains

The purity, biochemistry, virulence of *Escherichia coli* strains were determined by the conventional methods. The primary typical *Escherichia coli* strain was screened for further vaccine researches. The virulent *Escherichia coli* strain was chosen for the establishment of animal models (The detailed methods are shown in Supplementary file

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1).

2.7. Construction of the Ch-O111-1 mutant strain (Ch-O111-1/△galE)

To further extend the serological cross-reactivity with a variety of Gram-negative bacteria, the galE gene of Ch-O111-1 strain was deleted by homologous recombination according to the previous methods[31] which were briefly shown in Figure.2. Briefly, the recombinant suicide plasmid pCVD442/△galE was constructed by amplifying the up- and down- stream fragments by PCR using the indicated amplification primers in supplementary Table.1. and subcloning into the pCVD442 plasmid by restriction enzyme digestion (Sac Ⅰ and XbaⅠ) and ligation (T4 DNA ligase). The pCVD442/△galE plasmid was translated into competent cells DH5a, amplified, and further electrotransformated into the competent cells SM10. The SM10 (pCVD442/△galE) and Ch-O111-1 (NalR) strains were cultivated in LB at 37 ºC until the OD_{600} was about 0.8, mixed equivalently, and filtered into nylon membrane. The filter membrane was cultured at 37 ºC overnight on LA without antibiotic. The Ch-O111-1(pCVD442/△galE) was obtained by anti-nalidixic acid (Nal 50μg/ml) and anti-ampicillin (Amp 100μg/ml) screen of the 0.01mol/L $MgSO_4$ washed bacteria and designated Ch-O111-1/△galE.

2.8. Identification of the strain Ch-O111-1/△galE (Z9)

The galE gene fragments of Ch-O111-1 and Ch-O111-1/△galE (Z9) were determined by PCR and sequencing using the indicated identification primers in supplementary Tab.1. The LPS of Ch-O111-1 and Ch-O111-1/△galE (Z9) were extracted by the LPS Extraction Kit, separated by SDS-PAGE, and stained by the

DOI: 10.55415/deep-2022-0056.v1

Silver Stain Kit (Beyotime Institute of Biotechnology, China) according to the provided specifications. The bovine anti-Z9 serological cross-reactivity with a variety of clinical *Escherichia coli* strains was detected by ELISA.

2.9. Determination of virulence for mice and cows

The virulence of each candidate *Escherichia coli* strain was determined in 12-week-old Balb/c mice. The virulence of the mouse-lethal *Escherichia coli* strain was further assayed in 3-year-old lactational Holstein cows by determining the rectal temperature, electrical conductivity of milk (ECM, by Hand-held mastitis detection instrument, Type DRAMINSKI MA-1, Beijing Fubangshengye Science and Technology Co, Ltd), and bacteria concentration by the conventional methods. Especially, to establish the mouse and cow mastitis models, the virulence of the *Escherichia coli* LZ06 strain was determined in details in the mice and cows. Briefly, LD_{50} was determined by intraperitoneally (i.p.) injecting 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 (10 mice per group). The virulence of LZ06 was further assayed by intramammarily (i.m.) inoculating 500, 1000, or 2000 CFU per cow into 3-year-old lactational Holstein cows (5 cows per group).

2.10. Preparation of various Z9 vaccines

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

DOI: 10.55415/deep-2022-0056.v1

vaccine was prepared by the conventional methods, and the final Z9 concentration was 1×10^{10} CFU/ml. SP01-adjuvanted inactivated Z9 vaccine was prepared by the methods as described before[29, 30], and the final Z9 concentration was 1×10^{10} CFU/ml.

2.11. Mouse and cow protection assay

Six-week-old Balb/c mice (10 mice per group) were inoculated subcutaneously (s.c.) the indicated vaccines once every 14 days for 3 times. Then the immunized mice were challenged by injecting i.p. 3.8×10^{8} CFU per mouse 14 days post the third immunization for observing the survival rates. The sera were collected in the indicated time and determined by ELISA.

Three-year-old lactational Holstein cows (5 cows per group) were inoculated s.c. with the indicated vaccines once every 28 days for 3 times. Then the immunized cows were challenged by injecting i.m. 1000 CFU per cow 28 days post the third immunization for observing the clinical symptoms and determining the rectal temperature. The sera were collected in the indicated time and determined by ELISA. The milk was collected in the indicated time for determining ECM and bacteria concentrations.

2.12. ELISA

The presence of serum IgG specific to Z9 or the indicated clinical isolates was determined by indirect ELISA. Briefly, 0.1ml 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

DOI: 10.55415/deep-2022-0056.v1

serum samples assayed at a dilution of 1:25 from the unimmunized animals. The titer of each serum 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.

2.13 Statistics

All the quantitative data were expressed as the mean ± SD, performed by one way ANOVA analysis following Student's *t*-test using GraphPad Prism (version 5.0, GraphPad Software, CA). Survival curves were analyzed by a log rank test. A *P* value of < 0.01 was considered to be significant.

3. Results

3.1. The candidate vaccine or challenge strain for developing bovine coliform mastitis vaccine was obtained from lots of milk samples with clinical mastitis

The history of the candidate bovine mastitis vaccine strain was shown in supplementary Fig.1. Briefly, 344 coliform strains were isolated from 1321 milk samples, which were collected from cows with clinical mastitis from many dairy farms of the different regions in China. The serotype identification results showed that the ratios of O111, O2, O8, O21, O81, O86 and others in the isolated *Escherichia coli* strains were 47.1%, 11%, 12.2%, 6.7%, 10.5%, 6.9% and 5.5%, respectively. We randomly chose 7 typical O111 strains for further identification in details. By the conventional methods according to Bergey's Manual of Determinative Bacteriology, the biological characters of 7 O111 strains including Gram-staining, MCA culture,

DOI: 10.55415/deep-2022-0056.v1

biochemical test and purity identification were accordant with *Escherichia coli* (data not shown). Furthermore, we assayed the cross-reactivities of cow anti-Ch-O111-(1-7) antibodies with the isolated clinical strains by ELISA, the data in Tab.1 showed that Ch-O111-1 had a good cross-reactivity, which was all above 40%, which was higher than the other strains (data not shown). Thereafter, we chose the Ch-O111-1 strain for developing bovine coliform mastitis vaccine.

To obtain the candidate challenge strain for evaluating the protective efficacy of the vaccines against bovine coliform mastitis, LD_{50}s of 9 typical *Escherichia coli* strains were determined in 12-week-old Balb/c mice. They LD_{50}s were between 1×10^6 and 1×10^8CFU. Three mouse-lethal *Escherichia coli* strains were further assayed in 3-year-old lactational Holstein cows. The results showed that the *Escherichia coli* LZ06 strain could induce clinical mastitis in the infected cows, while the others could not (supplementary Fig.1). We further assayed the virulence of LZ06 in the mice and cows in details. The data in supplementary Fig.3 showed that the LD_{50} of LZ06 was 3.8×10^7 CFU, furthermore, more than 1000CFU LZ06 could induce the marked clinical mastitis in 3-year-old lactational Holstein cows, which was characterized by high rectal temperature, high bacteria concentration, low ECM. Thus, we established the mice model by i.p. infecting 3.8×10^8 CFU LZ06 into 12-week-old Balb/c mice and the bovine mastitis model by i.m. inoculating 1000CFU LZ06 into 3-year-old lactational Holstein cows.

3.2. The galE gene of Ch-O111-1 was knocked out, which was characterized by extensive cross-reactivity and attenuated virulence

DOI: 10.55415/deep-2022-0056.v1

As shown in supplementary Fig.2, 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. Meanwhile, the pCVD-galE u/d was constructed by enzyme digestion and ligation between pCVD442 and pMD18-T-galE-u/d. The pMD18-T-galE-u/d plasmid was constructed by ligation between the pMD18-T plasmid and up- and down- stream 250bp fragments of the galE gene. The up- and down- stream 250bp fragments of the galE gene were amplified by PCR with the indicated primers shown in Tab.3 and analyzed by electrophoresis. The correct pMD18-T-galE-u/d and pCVD-galE u/d plasmids were all screened and identified by restriction enzyme (Sac Ⅰ and Xba Ⅰ) digestion and electrophoresis, which were consistent with the design.

The data in Fig.1 showed that after anti-nalidixic acid and anti-ampicillin screen, two of nine randomly chose strains were Ch-O111-1 strains without the galE gene. Compared with 1634 bp PCR products in the 1-7 strains, there were only 524bp fragments in the 8 and 9 strains, which suggested that recombination happened in the 8 and 9 strains (Fig.1A). The silver-stained LPS extraction results showed that the LPS of the 8 or 9 strain was incomplete, which further verified the function of the galE gene in LPS synthesis (Fig.1B). We randomly chose the 9 strain for sequencing the galE gene. The data in Fig.1C showed that compared with the wild Ch-O111-1 strain, the underline 1110bp galE gene fragment of the 9 strain was deleted, which was designated Ch-O111-1/△galE (Z9).

To further explore the biological characters of Ch-O111-1/△galE (Z9), we determined its virulence in 12-week-old Balb/c mice and the cross-reactivity of the cow

DOI: 10.55415/deep-2022-0056.v1

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, which suggested that the virulence of Z9 was attenuated. As shown in Tab.1 and Tab.2, compared with the maternal strain Ch-O111-1, the mutant strain Z9 markedly extended the cross-reactivity ratio from about 50% to more than 90%, which suggested that Z9 maybe had extensive cross-protection.

3.3. Immunization of the different Ch-O111-1/△galE (Z9) antigens induced multiple levels of anti-Z9 antibodies in mice and cows.

To further strengthen the safety of Z9 for cows and environments, we chose the inactivated Z9 as antigens. For the inactivated antigens, the suitable adjuvant is important for the provocation of the optimal protection against infection. The data in Fig.2A and 2B showed 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 bovine coliform mastitis vaccine. The immunization regime is also the key factor for enhancing the protective efficacy of the vaccines against infection. As shown in Fig.2C and 2D, the anti-Z9 antibodies titers increased with boosting the vaccine dosages until 0.25ml in the mice and 5ml in the cows. The data in Fig2E and 2F showed that the anti-Z9 antibodies titers increased with boosting the inoculation times until three times.

Therefore, SP01-adjuvanted inactivated Z9 vaccine was s.c. inoculated 0.25ml into

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the mice once every 14 days for 3 times and 5ml into the cows once every 28 days for 3 times to evaluate the protective efficacy against *Escherichia coli* caused diseases.

3.4. Immunization of SP01-adjuvanted inactivated Z9 vaccine protected cows from LZ06 caused severe clinical mastitis and mice from LZ06 caused death

As shown in Fig.3, immunization of SP01-adjuvanted inactivated Z9 vaccines protected mice and cows from LZ06 caused diseases. Compared with the SP01 inoculated group, inoculation with SP01-adjuvanted inactivated Z9 vaccine markedly protected mice from LZ06 caused death. The protection efficacy even reached about 100% (Fig.3A). Furthermore, immunization with SP01-adjuvanted inactivated Z9 vaccine resulted in the improved clinical symptoms in the LZ06 challenged cows. Compared with the SP01 inoculated group, in which the rectal temperatures (Fig.3B), the ECM (Fig.3C) and the live bacteria concentrations (Fig.3D) of the challenged cows were all abnormal, SP01-adjuvanted inactivated Z9 vaccine protected the LZ06-challenged cows from severe mastitis.

Discussion and conclusion

In this study, a SP01-adjuvanted inactivated Z9 vaccine was developed, which could prevent the vaccinated cows from 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 reduced virulence. The inoculation regime of SP01-adjuvanted inactivated Z9 vaccine was formulated by optimizing the adjuvant, dosage and inoculation times and evaluated by the established animal models with the virulent clinical isolate LZ06 in the pre-clinical studies. Now,

DOI: 10.55415/deep-2022-0056.v1

the clinical trials of the SP01-adjuvanted inactivated Z9 vaccine were performed according to the guidelines of Agriculture Administration in China (NO.2014081).

Escherichia coli is one of the primary pathogens that caused bovine clinical mastitis according to our studies and the worldwide reports before[20, 32-34]. Commercial J5 bacterins were used in many countries for about 30 years, but there were many controversies about its protective efficacy against bovine mastitis[24-26, 28, 35-44], which may be attributed to the vaccine strain J5 that could not provide the valid protection all the time in the diverse countries that the different *Escherichia coli* prevailed[9, 34, 45, 46]. In China, the commercial J5 vaccine was used in the 2000's and disappeared in the market soon because its efficacy against bovine mastitis was often low in the clinical usage. Therefore, we screened the candidate vaccine strains by investigating the local clinical isolates. Now, the 'O111' antigen-group of *Escherichia coli* prevailed in China according to the serotype identification, which occupied 47.1% of the clinical isolates. So, we chose the 'O111' antigen-group of *Escherichia coli* for further vaccine strain screen.

Seven typical 'O111' strains were designated Ch-O111-(1-7) and their cross-reactivity was analyzed by ELISA. To avoid the false positive, the clinical isolate that its optical density mean value was more than 420 negative control mean value was designated reactivity positive. Among these 7 strains, Ch-O111-1 had the best extensive cross-reactivity that the positive ratios were between 40% and 65%, which further suggested that the J5 vaccine may have the low cross-reactivity with the isolates in China. Although Ch-O111-1 had the relative high cross-reactivity, it could not satisfied

DOI: 10.55415/deep-2022-0056.v1

the clinical need for developing the broad and effective mastitis vaccine against the diversity of *Escherichia coli* if the prevailed strains in the specific farms had not high cross-reactivity with Ch-O111-1. So, we chose the Ch-O111-1 strain for further reconstruction.

Lipopolysaccharide (LPS) is the outside layer of the outer cell wall in the Gram-negative bacteria and made of a variable oligosaccharide region linked to a conserved core polysaccharide and lipid A regions. Oligosaccharide represents the variable region that determines the bacterial serotype. However, core polysaccharide is highly conserved that 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 a Rc-mutant without uridine diphosphate galactose epimerase (galE), which has not the linkage between galactose and glucose in the core antige[42]. The ‘O’ exposure mutant may be a suitable antigen strain that can provide protective immunity against wide range of Gram-negative bacteria infection[45, 52]. To extend the cross-reactivity, the galE gene of the Ch-O111-1 strain was deleted by homogenous recombination. Many tools have been described for producing the 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, which was

DOI: 10.55415/deep-2022-0056.v1

characterized by reduced virulence and extensive cross-reactivity (more than 90%) with many different serotypes of *Escherichia coli*. Therefore, the Ch-O111-1/ΔgalE strain was used for the development of bovine mastitis vaccine.

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

To evaluate the protective efficacy of the SP01-adjuvanted inactivated Z9 vaccine against bovine mastitis, the suitable animal models were established with the isolate LZ06 in 12-week-old mice and 3-year-old Holstein cows in this study. Although the mouse-lethal model was not suitable for evaluating the protective efficacy of the vaccine against bovine mastitis, it could provide the reference of the quality of the vaccine in the pre-clinical studies at the least cost. The established Holstein cow model was consistent with the reported data before[36, 37], which showed that the rectal temperatures, bacteria concentrations and so on were abnormal. Although the more CFUs of bacteria infected, the severer the clinical mastitis happened in the cows, the animal ethics and bio-safety was not permitted. In these established animal models, the SP01-adjuvanted inactivated Z9 vaccine markedly prevented the infected animals from the severe diseases.

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In conclusion, the SP01-adjuvanted inactivated Z9 vaccine was successfully developed, which could protect the cows against bovine clinical mastitis caused by *Escherichia coli.*

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Conflict of Interests

The authors declare that they have no any conflict of interests.

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DOI: 10.55415/deep-2022-0056.v1

Tab.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 1×10^8CFU/ml inactivated clinical bacteria isolates as described in the text. The reactivity of cow anti-Ch-O111-1 antibodies was determined by ELISA. The clinical isolate that its optical density mean value was more than 420 negative control (Healthy Holstein cow serum) mean value was designated reactivity positive.

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Tab.2. 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 1×10^8CFU/ml inactivated clinical bacteria isolates as described in the text. The reactivity of cow anti-Z9 antibodies was determined by ELISA. The clinical isolate that its optical density mean value was more than 420 negative control (Healthy Holstein cow serum) mean value was designated reactivity positive.

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Supplementary Tab.1. Primers used for generation and determination of Z9

Primer name	**Primer sequence,5'-3'**
galE-u-F(F1), amplification	TCGGACGGTGGGCTCTATCGCTATG
galE-u-R(R1), amplification	TGTAGGCCGGAGAGGGGGCTTACGC
galE-d-F(F2), amplification	GGGCGGACGCCAGCAAAGCCGACCGTGAA
galE-d-R(R2), amplification	GCAACGCCATCAAAGGATCGTTGCT
galE-R(F3), identification	TCGGACGGTGGGCTCTATCGCTATG
galE-F(R3), identification	GCAACGCCATCAAAGGATCGTTGCT

DOI: 10.55415/deep-2022-0056.v1

Supplementary Tab.2. Key resources table

Reagent	Source	Identifier
$Al(OH)_3$	Brenntag Biosector, Denmark	21645-51-2
Duoprime PenReco	Calumet, USA	N/A
SP01 adjuvant(Duoprime PenReco)	Beijing Institute of Microbiology and Epidemiology	N/A
Lysogeny broth (LB)	BD, USA	244620
Lysogeny agar (LA)	BD, USA	244520
Tryptic soy broth (TSB)	BD, USA	211825
Tryptic soy agar (TSA)	BD, USA	212305
MacConkey agar (MCA)	BD, USA	211390
The Escherichia coli serotype determination kits	Japan Biology Institute and Denmark Statens Serum Institute Co., LTD, Japan.	N/A