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Efficacy of enterotoxigenic *Escherichia coli* vaccine for bovine clinical mastitis

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An enterotoxigenic *Escherichia coli* (ETEC) vaccine designed to prevent diarrhoea was inoculated into dairy cows, and the occurrence of clinical mastitis was investigated for 2 years. Half of 480 cows in five farms were subcutaneously inoculated with ETEC vaccine (Imocolibov) twice with a 1-month interval in 2007 and 2008. Fisher's exact test and survival (time to event) analysis with the log-rank test were used to compare vaccinates and controls. In 2007, there was no significant difference in the incidence rate of mastitis between vaccinate (20·3%) and control (17·1%) cows. The rate of death or culling due to mastitis was lower in vaccinated cows (7·4%) than in control cows (29·2%, $P=0·07$, Fisher's exact test; $P=0·02$, log-rank test). In 2008, there was no significant difference in both the incidence rate of mastitis and the rate of death or culling due to mastitis. Milk productivity was compared between vaccinates and controls in one farm. Multi-way analysis of variance (ANOVA) was performed for the amount of 4% fat-corrected milk, and there was no significant difference between vaccinates and controls. These results suggest that ETEC vaccine inoculation reduces death or culling due to mastitis, whereas no preventive effect on the development of mastitis was observed.

Keywords: Vaccine, bovine mastitis, ETEC, efficacy.

Mastitis is an inflammation of the mammary glands and is an important cause of milk production loss for dairy farms. Among the many causative bacteria, coliform mastitis induces systemic symptoms and leads to death or inhibition of milk secretion.

In Europe and North America, mastitis vaccines developed from *Escherichia coli* O111:B4 strain J5 (Overbeek et al. 1987) or *Salmonella typhimurium* Re-17 mutant (McClure et al. 1994) have been investigated for their efficacy (Hogan & Smith 2003). Wilson et al. (2007) reported that immunization with J5 was associated with protection against severe clinical coliform mastitis signs, culling, and death from clinical mastitis.

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In contrast, another coliform vaccine (Imocolibov; Merial, Scientific Feed Laboratory Co., Ltd., Tokyo, Japan) designed for the prevention of diarrhoea in neonatal calves, is widely used because no mastitis vaccine is available in Japan. This vaccine is used to inoculate not only breeding beef cattle, but also dairy cattle. This vaccine contains inactivated bacterial cells expressing virulence factor K99, Y, and 31A, and serotype O78. Specific antibodies against fimbrial and somatic antigens are produced in the blood of immunized animals. When this vaccine is used to inoculate pregnant cows, these antibodies are transferred to neonatal calves via colostrum, and reduce diarrhoea and mortality caused by enterotoxigenic *Esch. coli* (ETEC) strains in calves; therefore, this vaccine may be used to prevent mastitis (Mizobuchi et al. 1997). Mizobuchi et al. (1997) compared the prevalence of mastitis between farms inoculated with the ETEC vaccine and unvaccinated control farms, and

compared before and after vaccination; however, prevalence was not compared between vaccinated and control cows in the same farm and the analysis could have excluded the effect of individual farm factors. The present study was undertaken to examine the effect of diarrhoea vaccine on the prevalence of mastitis in dairy cows with consideration of the farm factor.

Materials and Methods

Animals

We selected dairy farms in which death or culling due to coliform mastitis occurred. Four farms (A, B, C and D) in 2007 and three farms (A, C and E) in 2008 were selected. Farms A and B were compost barns, and farms C, D and E were tie stalls using sawdust for bedding material. Two-hundred-and-seventy-three (A:152; B:24; C:41; D:56) cows in 2007 and 207 (A:141; C:40; E:26) in 2008 were used. Cows for culling, non-pregnant heifers and unhealthy cows were excluded. There was no difference in the age of these cows and between vaccinates and controls.

Vaccination

The ETEC vaccine used in this study (Imocolibov; Merial, Scientific Feed Laboratory Co., Ltd.) was an inactivated aluminum hydroxide and saponin-added vaccine against neonatal colibacillosis of calves and lambs, supplied as a suspension for injection. It contains a formalized culture of *Esch. coli* strains associated with neonatal diarrhoea and septicaemia in calves and lambs, and formaldehyde. Somatic serotypes of *Esch. coli* strains were O9, O8, O15, O78, O101 and O117 expressing virulence factors K99 ($\geqslant$ 1·22 SA. U), Y ($\geqslant$ 1·80 SA. U), 31A ($\geqslant$ 1·27 SA. U); serotype O78 ($\geqslant$ 1·14 optical density Units). '1 SA. U' means an adequate dose to obtain an agglutinating antibody titre of 1 log 10 in guinea-pigs after two administrations of vaccine. This ETEC vaccine was subcutaneously inoculated into half of the cows at each farm at the same time into the neck or rear leg, and additional inoculation was performed 1 month later. The remaining half of the cows did not receive treatment. The number of vaccinated cows was 133 in 2007 (A:70; B:13; C:20; D:30) and was 102 in 2008 (A:67; C:20; E:15). First inoculation was conducted from May to July. Because coliform mastitis occurs frequently in summer (Hogan & Smith 2003), the vaccine was administered at the beginning of summer.

Definition of mastitis

Clinical mastitis treated by veterinarians affiliated with veterinary clinical centers was defined as 'mastitis'.

Research into the incidence of mastitis was based on the medical records of veterinarians. 'Incidence rate' and 'rate of death or culling' are defined as follows:

Incidence rate (%) is the percentage of the number of cows with clinical mastitis relative to the total number of cows examined. Rate of death or culling (%) is the percentage of the number of cows with death or culling relative to the number of cows with clinical mastitis. Mastitis associated with severe systemic symptoms, such as high fever, thrombocytopenia, hyperaemia of palpebral conjunctive, motility stopping of rumen, and poor appetite was defined as 'peracute mastitis'.

Milk sample collection and isolation of bacterial pathogen

Milk samples from mastitis cows were collected from the time of the first inoculation to the end of December from farm A in 2007, and from farms A, C and E in 2008. Milk samples were aseptically obtained once during the first medical care. These samples were cooled to 4 °C or frozen at −20 °C and transported to our laboratory. Samples were diluted tenfold with distilled water and original and diluted samples were inoculated at 50 µl each to 5% Sheep Blood Agar (Eiken Chemical Co., Ltd., Tokyo, Japan), Baird-Parker RPF agar (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan) and desoxycholate-hydrogen sulphide-lactose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). These media were cultured at 37 °C under aerobic conditions, and incubation time was 24 h. Isolated bacteria were classified into four groups as enterobacteria, *Staphylococcus* sp., *Streptococcus* sp. and other organisms. For identification of enterobacteria, we used a test kit, such as Api20E or rapid 20E (SYSMEX bioMérieux Co., Ltd.).

Milk yield

The milk yield was compared between vaccinates and controls at farm A based on the cattle herd test. Milk yield data were obtained once a month and 4% fat-corrected milk (FCM) was calculated from the equation: 4% FCM (kg) = 0·4 × milk yield (kg) + 15 × fat yield (kg). Primiparous cows were excluded from data analysis. Total milk production data were 1535.

Statistical analysis

Statistical analysis was performed with the computer software R version 2.10.1 for Windows OS (Comprehensive R Archive Network , http://cran.r-project.org/). An additional package used was 'survival' (Author: Terry Therneau).

For comparison of the incidence of clinical mastitis and peracute mastitis among all vaccinates or controls, the entire population of study cows was tested. For comparison of the rate of death or culling due to mastitis between vaccinates and controls, only the population of cows with clinical mastitis was tested. In the same way, the rate of death or culling due to peracute mastitis was compared.

We used Fisher's exact test to compare the incidence of mastitis and the rate of death or culling between vaccinates and controls. Survival (time to event) analysis was also used. The Kaplan-Meier method with the log-rank

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Table 1. Comparison of incidence of mastitis between vaccinates and controls in 2007 and 2008

	No. (%) of cows of each group in 2007		No. (%) of cows of each group in 2008	
Mastitis	Vaccinates	Controls	Vaccinates	Controls
Peracute	7 (5·3)	6 (4·3)	8 (7·8)	5 (4·8)
Other clinical	20 (15·0)	18 (12·9)	19 (18·6)	21 (20·0)
Total	27 (20·3)	24 (17·1)	27 (26·5)	26 (24·8)

test was used to compare survival curves between vaccinates and controls.

Because the farms investigated in 2008 were different from those investigated in 2007, analysis for the two years was performed separately. A P value $<0{\cdot}05$ was considered significant. For comparison of the isolation rate of aetiologic agents of mastitis, Fisher's exact test was used.

The lactation performance of farm A was analysed using the lactation data of multiparous cows. These data were classified by days from vaccination and days in milk. The categories of days from vaccination were −100–0, 1–100, 101–200 and 201–300 d. The categories of days in milk were 0–100, 101–200, 201–300 and 300 d and over.

Multi-way analysis of variance (ANOVA) was performed on the 4% FCM as a dependent variable. Independent variables were individual cows, vaccination status, category of days from vaccination and category of days in milk.

The analysis model was MILK = COW + VAC + CDV + CDM + e, where MILK is 4% FCM; COW is individual cow; CDV is category of days from vaccination; CDM is category of days in milk; VAC is vaccination status and e is unexplained variation.

Multi-way ANOVA was also performed on the 4% FCM in each category of days in milk. Independent variables were individual cows, vaccination status and category of days from vaccination.

The analysis model was MILK = COW + VAC + CDV + e, where MILK, COW, VAC, CDV and e have the same meaning as above.

Results

Generation status of mastitis in 2007

The total number of clinical mastitis cases on four farms was 51 (27 vaccinates and 24 controls) and that of peracute mastitis cases was 13 (7 vaccinates and 6 controls; Table 1). There was no significant difference in the incidence of mastitis ($P=0{\cdot}54$, Fisher's exact test) or peracute mastitis ($P=0{\cdot}78$) with the vaccination status.

The rate of death or culling due to mastitis in vaccinates (7·4%) had a tendency to be lower than that of controls (29·2%) ($P=0{\cdot}07$, Fisher's exact test; Table 2). Survival curves were significantly different between vaccinates and controls ($P=0{\cdot}02$, time to event analysis, log-rank test; Fig. 1). The numbers of death or culling with peracute mastitis was 6, 1 vaccinate (14·3%) and 5 controls (83·3%). There were significant differences in the rate of death or culling due to peracute mastitis with the vaccination status ($P=0{\cdot}03$, Fisher's exact test, log-rank test). In contrast, the rate of death or culling due to mastitis except peracute mastitis had no significance with the vaccination status ($P=0{\cdot}59$, Fisher's exact test; $P=0{\cdot}29$, log-rank test).

Table 2 Comparison of rate of death or culling due to mastitis between vaccinates and controls in 2007 and 2008

	No. (%) of cows of each group in 2007		No. (%) of cows of each group in 2008	
Mastitis	Vaccinates	Controls	Vaccinates	Controls
Peracute	1 (14·3)*	5 (83·3)	1 (12·5)	0 (0)
Other clinical	1 (5·0)	2 (11·1)	0 (0)	0 (0)
Total	2 (7·4)	7 (29·2)	1 (3·7)	0 (0)

*Significantly different from controls ($P=0{\cdot}03$, Fisher's exact test).

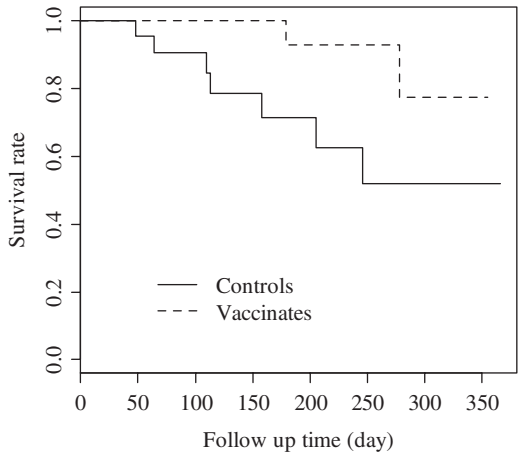

Fig. 1. Kaplan-Meier survival curves of mastitis cows affected in 2007. The survival rate of vaccinates was significantly greater than that of controls ($P=0{\cdot}02$, log-rank test).

Table 3 shows the pathogens isolated from 26 mastitis cases. There was no significant difference in the isolation rate of *Esch. coli* between vaccinates and controls.

Generation status of mastitis in 2008

The total number of clinical mastitis cases on three farms was 53 (27 vaccinates and 26 controls) and that of peracute mastitis cases was 13, 8 vaccinates (7·8%) and 5 controls (4·8%; Table 1). The number of deaths or culling due to mastitis was 1 and that due to peracute mastitis was 1 vaccinate (Table 2). There was no significant difference according to the vaccination status. The rate of death or culling due to mastitis had no significant difference according to the vaccination status. There was no significant difference in the incidence of clinical mastitis between 2007 and 2008; however, the number of death or culling in 2008 was significantly lower than in 2007.

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Table 3. Percentage of aetiologic agents isolated from clinical mastitis cases

	2007 (%)†		2008 (%)‡	
	Vaccinates	Controls	Vaccinates	Controls
Escherichia coli	17	21	10	25
Klebsiella pneumoniae	0	0	15	0
Other enterobacteria	8	0	15	0
Staphylococcus sp.	17	21	10	8
Streptococcus sp.	25	7	10	42
Other microorganisms	0	14	5	8
No pathogen or mixed pathogen isolated	33	36	35	17

† Percentage of cows infected with each pathogen in 2007 isolated from farm A.
‡ Isolated from farms A, C and E.

Table 3 shows the pathogens isolated from 32 cases. Similarly to the above, there was no significant difference in the isolation rate of *Esch. coli* between vaccinates and controls. *Klebsiella pneumoniae* was isolated from farms C and E only.

Lactation performance of farm A

Multi-way ANOVA on the 4% FCM showed that it was significantly different according to cows, category of days from vaccination and category of day in milk ($P<0{\cdot}05$); 4% FCM yield of vaccinates had a tendency to be larger than that of controls ($P=0{\cdot}051$).

Multi-way ANOVA on 4% FCM in each category of day in milk revealed that it was not significantly different.

Discussion

From the results in 2007, the survival rate of vaccinates was higher than that of controls, although there was no significant difference in the incidence rate of mastitis between the two groups. It was suggested that inoculation of the ETEC vaccine was associated with a lower rate of death or culling than in control cows. This is similar to a previous study that reported a lower rate of death or culling among ETEC-vaccinated farms (Mizobuchi et al. 1997). It is suggested that the ETEC vaccine used in this study would be useful against mastitis; however, in 2008, there was no significant difference in the rate of death or culling due to mastitis between vaccinates and controls. This difference may have been caused by the low number of mastitis cases associated with death or culling in 2008. It is possible that the severity of *Esch. coli* mastitis is mainly determined by cow factors (Burvenich et al. 2003). Because the investigation in 2008 was performed using surviving cows from the 2007 study and some primiparous cows, these cows might have developed resistance properties against severe mastitis. On the other hand, aetiologic agents of mastitis were similar for both 2007 and 2008 studies, and environmental or cow factors were associated with prevalence. Consequently, assuming that severe coliform mastitis is more likely to occur, for example, when sensitive cows are infected, the advantageous effect of ETEC vaccine might become apparent. In addition, mastitis incidence and the rate of death or culling due to mastitis varied from farm to farm. The appropriateness of vaccination should be assessed with consideration of the generation status of mastitis in individual farms.

The mechanism of reduction of death or culling due to mastitis using ETEC vaccination is unknown. On the other hand, the mechanism of J5 vaccination against bovine coliform mastitis has been hypothesized. Traditionally, increased serum and milk antibody titres used against *Esch. coli* J5 are considered effective protection against coliform mastitis by enhanced opsonization of lipopolysaccharides (LPS) and bacteria (Tyler et al. 1988). Dosogne et al. (2002) reported that J5 vaccination may reduce the severity of coliform mastitis by inducing mammary gland hyperresponsiveness, characterized by a T helper 1 (Th1) response and mediated by memory cells inside the mammary gland.

The ETEC vaccine consists of 6 strains of *Esch. coli* which have pili antigens, such as K99, Y, 31A, or serotype O78. A specific antibody is produced in the blood following ETEC vaccine administration, and secreted into the colostrum. The LPS structure of Gram-negative smooth bacteria consists of a polysaccharide side chain (O antigen), R-core polysaccharides and lipid A. The core antigen compounds are highly conserved among Gram-negative bacteria. Antibody against *Esch. coli* J5 cross-reacts with core antigens of heterologous Gram-negative LPS (Tyler et al. 1992). We also found that the antigen of ETEC vaccine cross-reacted serologically against *Esch. coli* J5 strain (Morimoto et al. 2009). The correlation between the antibody titre against ETEC vaccine and the severity of mastitis will be investigated in the future.

In this study, vaccination was not associated with the incidence rate of clinical mastitis or peracute mastitis. For this reason, this ETEC vaccine might save cows with severe symptoms associated with clinical mastitis, but will not prevent mastitis infection; however, Mizobuchi et al. (1997) reported that the incidence of mastitis in vaccinated farms was lower than that in unvaccinated farms. With J5 vaccine,

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González et al. (1989) reported that administration of the *Esch. coli* J5 vaccine reduced the incidence of clinical Gram-negative mastitis, whereas Wilson et al. (2007) reported that immunization with J5 was not associated with any reduction in overall clinical mastitis. These discrepancies may be related to the nature of the pathogenic agent.

In conclusion, inoculation with ETEC vaccine was associated with reduction of the rate of death or culling due to clinical mastitis, but not with the incidence of clinical mastitis. This vaccine can be used for the prevention of mastitis as well as diarrhoea.

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