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Survival of *Escherichia coli* harboring nucleic acid-hydrolyzing 3D8 scFv during RNA virus infection

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ABSTRACT

Previously, *Escherichia coli* harboring the codon-optimized *3D8scFv* gene (*E. coli* 3D8scFv) was developed as a feed additive for use in preventing norovirus infection. Here, we evaluated whether the *3D8scFv* gene affects the colonization of *E coli* when *E. coli* 3D8scFv passes through the mouse gastrointestinal tract. To determine the colonization ability of *E. coli* 3D8scFv, *E. coli* cells with or without the *3D8scFv* gene were fed to mice. Total DNA was extracted from the animals' stools, stomach, small intestine and colon. All samples were amplified using *3D8scFv* gene-specific primer sets. *E. coli* 3D8scFv begins to be excreted 1 h after feeding and that all *E. coli* 3D8scFv cells were excreted between 12 and 24 h after the last feeding of the cells. The previously measured gastrointestinal transit time of the mice was between 8 h and 22 h. The results of this study therefore show that *E. coli* 3D8scFv cannot colonize the gastrointestinal tracts of mice. In addition, if the purified 3D8 scFv protein is used as a feed additive, any associated *E. coli* 3D8scFv bacteria will not colonize the gastrointestinal tracts of the livestock. Thus, this feed additive meets the safety assessment criteria for the commercial use of bacteria.

1. Introduction

Humans have been making efforts to combat novel diseases caused by rapidly evolving viruses. Recently, avian influenza A (H7N9) viruses from East Asia have spread to the Americas and Europe, and human infection with avian influenza A virus has been reported in China (Artois et al., 2017). In addition, Ebola virus and beta coronavirus, which cause Ebola hemorrhagic fever and Middle East respiratory syndrome (MERS), respectively, are known to be fatal to humans. These viruses have spread to Africa, the Middle East, and Asia and have raised the fear of new viruses (Mackay and Arden, 2015; Martinez et al., 2015). Notably, some viruses isolated from animals have been reported to induce fatal diseases in humans due to the mutation and evolution of the animal viruses (Parrish et al., 2008). Avian influenza A viruses from birds, Ebola viruses from bats and rodents, and beta coronaviruses from bats and camels have been transmitted to humans (Parrish et al., 2008). All of these viruses are single-stranded RNA viruses. Therefore, the prevention of virus infections in livestock is essential for eliminating the potential risk to human life caused by cross-species viral transmission between humans and livestock.

Nucleic acid antibodies are significantly increased in patients with autoimmune diseases (Marion et al., 1997). The antigens recognized by most of these anti-nucleic acid antibodies have been shown to be single- or double-stranded DNA or RNA molecules that contain no specific common target sequences; dinucleotides such as dTdT and dGdC have also been reported as antigens of these antibodies (Kim et al., 2006).

The nucleic acid antibody 3D8 was isolated from the spleen cells of mice with autoimmune syndrome (Kwon et al., 2002). The VH and VL of the mAb 3D8 IgG are connected by a flexible linker, $(G_4S)_3$, to create 3D8 scFv (Kim et al., 2006). This recombinant single-chain variable fragment can penetrate cells via caveolae-mediated endocytosis. Once endocytosed, 3D8 scFv remains in the cytoplasm without further trafficking into endosomes, lysosomes, the endoplasmic reticulum, the Golgi, or the nucleus. In the nucleus, 3D8 scFV induces the hydrolysis of both DNA and RNA (Jang et al., 2009). 3D8 scFv has also been reported to exert an antiviral effect against classical swine fever virus (CSFV), which is an RNA virus that primarily infects pigs (Jun et al., 2010). A strain of *Escherichia coli* harboring nucleic acid-hydrolyzing codon-optimized 3D8 scFv (*E. coli* 3D8scFv) was developed for use as a feed additive to prevent norovirus infection, and *Lactobacillus paracasei*

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harboring nucleic acid-hydrolyzing 3D8 scFv was developed for use as a preventive probiotic. The use of these strains was shown to prevent the induction of apoptosis during murine norovirus (MNV) infection and to decrease mRNA expression for the viral capsid protein VP1 (Hoang et al., 2015).

Genetically modified microorganisms must be evaluated for safety before use as commercial food or as food additives for humans or livestock, because genetically modified microorganisms may produce toxic materials and allergens and it can also destruct the microbial ecosystem. The test of the colonization of genetically modified microorganisms is also the main issue in human safety, because the colonization can affect microbiome of human or livestock microbiome negatively. For assessing the risk of genetically modified organisms, the concept of substantial equivalence has been adopted by the Organization for Economic Co-operation and Development (OECD) and is used globally. According to this concept, *E. coli* 3D8scFv should not have any effect, except an anti-viral effect, on the animals to which it is administered. For example, oral ingestion of *E. coli* 3D8scFv must not induce changes in weight or mortality in comparison to oral ingestion of control *E. coli*.

Here, we evaluated whether *E. coli* 3D8scFv colonizes the gastrointestinal tracts of mice. To comply with the substantial equivalence concept that is required for safety assessment when purified 3D8 scFv protein is used as a feed additive, we also tested whether ingested *E. coli* 3D8scFv are excreted rapidly.

2. Material and methods

2.1. Bacterial strains

E. coli BL21 (DE3) (*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*) harboring a codon-optimized *3D8scFv* gene in pET42 was used to express 3D8 scFv in *E. coli*. This *E. coli* strain was used both as an animal feed and in the protein purification reported in this study. The pET42 vector was cleaved by the DNA restriction enzymes *NdeI* and *XhoI* to remove the GST tag. *E. coli* BL21 (DE3) was used in animal feed as a control.

2.2. Cell growth curves and colony-forming unit (CFU) assays

E. coli BL21 (DE3) harboring either pET42-*3D8scFv* or empty pET-42 lacking the GST coding region was streaked on M9 plates containing 50 μg/ml of kanamycin and incubated at 37 °C. Three colonies from each plate were separately cultured in M9 liquid medium containing 0.4% glycerol at 37 °C for 12 h. After this time, 3 ml of the cell culture was used to inoculate 100 ml of fresh M9 liquid medium, and the starting OD_{600} of the culture was measured (0 min). Aliquots of the cell cultures were collected at various time intervals (1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d), and the OD_{600} of the culture and the number of CFUs present were measured. To calculate the number of CFUs present in cultures of *E. coli* BL21 (DE3) containing either pET42-*3D8scFv* or pET-42, the collected cells were washed three times with saline and spread on M9 plates containing 50 μg/ml of kanamycin. The plates were incubated for 18 h at 37 °C, and the number of colonies per ml of cell suspension plated was counted.

2.3. Protein turnover assay

pET42-*3D8scFv* was transformed into *E. coli* BL21 (DE3) cells, and the cells were incubated at 37 °C. When the OD_{600} of the culture reached 0.6, 0.5 mM isopropyl-β-D-1-thiogalactoside (IPTG) was added to induce the expression of the 3D8 scFv protein, and aliquots of the cell cultures were collected 0, 3, 6, 12, 24 and 48 h after induction. The collected cells were lysed by sonication in lysis buffer (50 mM $Na_2H_2PO_4$, 500 mM NaCl, 10 mM β-mercaptoethanol, pH 8.0) and centrifuged at 12,000 rpm for 10 min at 4 °C. The proteins present in the cell pellets were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene difluoride) membranes. The blots were probed with a polyclonal anti-His tag antibody (#2365, Cell Signaling Technology, Danvers, MA, USA) and with a monoclonal anti-OmpF antibody (orb13626, Biorbyt, San Francisco, CA, USA).

2.4. Animals and diets

A total of twenty-five male specific pathogen-free (SPF) ICR mice aged 8 weeks were used. The animals were purchased from DBL Co., Ltd. (Umsung, Korea). The mice were maintained in an SPF facility under a 12 h light-dark cycle (lights on 6:00; lights off 18:00) at 22 ± 0.5 °C and 40–60% relative humidity and were allowed free access to chow (Teklad Global 18% Protein Rodent Diet (cat. #2018S), Harlan Laboratories Inc., WI, USA) and water. All experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Korea Research Institute of Bioscience and Biotechnology (approval No: KRIBB-AEC-16145).

2.5. Experimental design

The mice were acclimatized and fasted for 24 h. Supplemented drinking water and feed were changed daily. Mortality and individual clinical signs were also assessed daily. *E. coli* with or without the *3D8scFv* gene ($2x10^9$ cells/time) or saline was fed to individual groups of mice three times at 24-h intervals. The mice were weighed, and stools were collected at various time intervals (0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 6 d, 15 d, 18 d, and 21 d) after the last feeding of either cells or saline. After 21 days, the animals were sacrificed, necropsies were performed, and total DNA was extracted from the stomachs, small intestines, and colons of the animals.

2.6. Bacterial survival study

Total DNA was extracted from the stools obtained at each time interval (1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d) after the last feeding of either cells or saline. For bacterial cultures on plates, the stool from each time interval was dissolved in saline and centrifuged, and the supernatants were applied to the culture plates. Total DNA was extracted from the stomachs, small intestines, and colons of all mice sacrificed 21 days after the last feeding. The DNA samples were amplified using *3D8scFv* gene-specific primer sets and electrophoresed on 1.5% agarose gels.

2.7. Statistics

The OD_{600} values and CFU/ml values obtained for *E. coli* 3D8scFv were normalized to the values obtained for the control (**$p < 0.001$, *$p < 0.05$ for the comparison of the control with *E. coli* 3D8scFv). The *p* values for the comparison to the control were determined using a two-tailed *t*-test. The error bars indicate the SEM; n = 3 experiments. The mean body weights of the animals in the three experimental groups (mice fed *E. coli* 3D8scFv, mice fed control *E. coli* pET42 without a GST tag, and mice that received no treatment) were normalized to that of the control group (**$p < 0.001$, *$p < 0.05$ comparing the control to *E. coli* 3D8scFv or to no treatment). The *p* values were determined via one-way ANOVA followed by Dunnett's multiple comparisons test vs. the control for each time point. The error bars indicate the SEM; n = 10 experiments for the *E. coli* 3D8scFv and control groups and n = 5 experiments for the "no treatment" group.

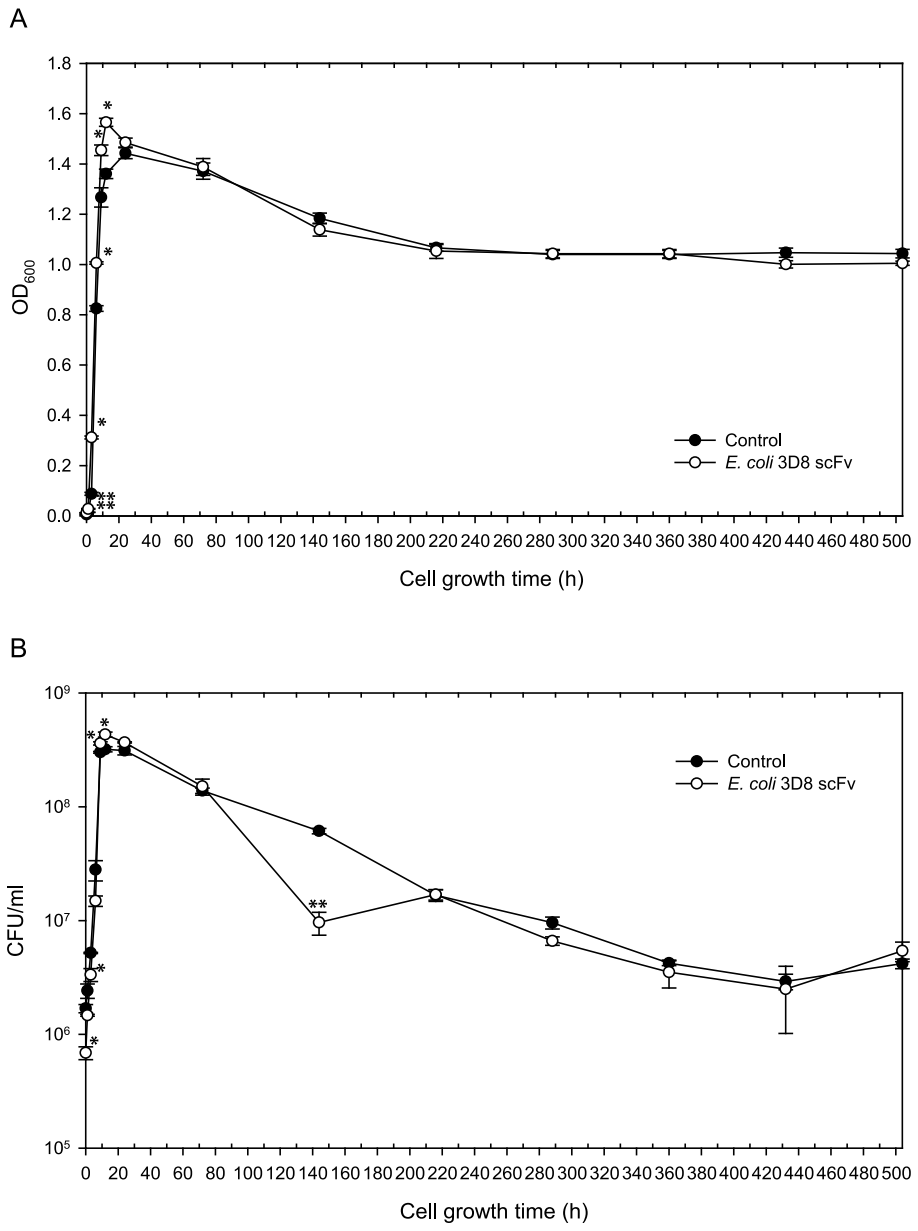

Fig. 1. Growth curves of *E. coli* 3D8 scFv. Growth curves of *E. coli* 3D8scFv and the control (*E. coli* pET42 without a GST tag) were generated using OD_{600} measurements (A) and counting of CFU/ml (B) at 12 time points (0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d) after inoculation of the cells into fresh medium.

3. Results

3.1. Effect of 3D8 scFv on E. coli 3D8scFv cell growth

The survival of *E. coli* 3D8scFv in mice may depend on the strain's ability to colonize the gastrointestinal tract. However, the possibility that *E. coli* 3D8scFv exhibits an altered growth rate should be considered when measuring this survival rate. Therefore, before injecting *E. coli* 3D8scFv into mice, we analyzed the growth rate and the number of CFUs in *E. coli* 3D8scFv cultures grown in liquid medium. *E. coli* pET42, which contains the same genes as *E. coli* 3D8scFv except for the *3D8scFv* gene, was used as a control. M9 minimal medium was used to minimize the effects of the presence of varying levels of nutrients in the growth medium. Aliquots of fresh medium were inoculated with *E. coli* 3D8scFv or with the control strain, and the OD_{600} and the number of CFUs in the inoculated cultures were measured. Aliquots of the cell cultures were collected at the same time intervals (0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 6 d, 15 d, 18 d, and 21 d) used for the collection of stools in mice, and the OD_{600} and CFU of the cell culture aliquots were measured (Choi et al., 2017). The OD_{600} values of the *E. coli* 3D8scFv culture samples were measured and normalized to the blank value (Fig. 1A). To identify statistically significant differences between *E. coli* 3D8scFv and the control, p values were determined by using a two-tailed t-test versus the control for each time point (Park et al., 2017). The growth of both *E. coli* 3D8scFv and the control strain reached stationary phase after 1–3 h of lag phase growth and 9 h of exponential phase growth. The growth of *E. coli* 3D8scFv appeared to be slower than that of the control at 12 h, and that difference was statistically significant. However, the observed effect may be due to a difference in the number of cells in the inoculum. The OD_{600} values of *E. coli* 3D8scFv and the control were 0.0057 and 0.0127, respectively. To measure the viability of the *E. coli* 3D8scFv and control strains, we measured the CFUs present in the cultures (Fig. 1B). Both strains showed the highest CFU counts after a 12 h incubation; at 21 d, the number of CFUs had decreased but remained significant. This finding indicates that *E. coli* 3D8scFv can colonize the gastrointestinal tracts of mice if the

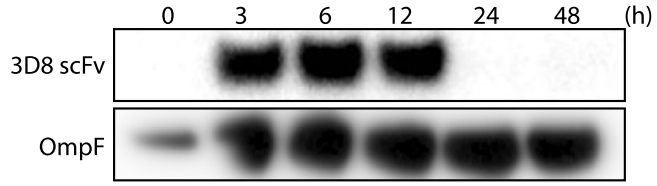

Fig. 2. Degradation of the 3D8 scFv protein. Western blotting assays were performed to determine the stability of the 3D8 scFv protein. pET42-*3D8scFv* was transformed into *E. coli* BL21 (DE3) cells. Aliquots of the cell cultures were collected 0, 3, 6, 12, 24 and 48 h after induction. The blots were probed with an anti-His tag antibody and with an anti-OmpF antibody.

appropriate conditions are present. For both strains, the CFU values and the OD_{600} values of the cultures exhibited statistically significant differences at 12 h; interestingly, the CFU value at 6 d also showed a statistically significant difference, indicating that the presence of the *3D8scFv* gene temporarily affects the viability of the cells.

3.2. 3D8 scFv protein degradation in vivo

E. coli 3D8scFv contains the *3D8scFv* gene fused with an N-terminal *phoA* signal peptide that leads to the secretion of the 3D8 scFv protein. To determine how long the 3D8 scFv protein remains in the cell, we used western blotting to analyze the rate of protein loss from cell cultures (Fig. 2). When the OD_{600} value of the cultures reached 0.6, the expression of the 3D8 scFv protein was induced; samples of the cell cultures were collected 0, 3, 6, 12, 24, and 48 h after induction and subjected to western blotting. The blots were probed with an anti-His tag antibody, and the expression of the 3D8 scFv protein was compared with the level of expression of the protein product of the *E. coli* housekeeping gene *ompF*. In a previous report, the plasma half-life of the scFv protein was shown to be 0.6 h (Hutt et al., 2012), and the stability of the protein was shown to depend on the amino acid sequence and the length of the linker between the VH and the VL of mAb 3D8 IgG. The results obtained in our study showed that the 3D8 scFv protein is stable for 12 h, after which it is rapidly degraded. Therefore, the 3D8 scFv protein can exist in mice for more than 12 h, even after all *E. coli* 3D8scFv has been excreted.

3.3. Survival of E. coli 3D8scFv extracted from stools

Next, we evaluated the ability of *E. coli* 3D8scFv to colonize the gastrointestinal tracts of mice. *E. coli* cells containing or lacking the *3D8scFv* gene ($2x10^9$ cells/sample) or saline were fed to mice (10 mice were fed *E. coli* 3D8scFv, 5 mice were fed host *E. coli*, and 5 mice were given saline) three times at 24-h intervals. The body weights of the mice were measured and stools were collected 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d after the last feeding of cells or saline (Fig. 3A). Until 24 h after the last feeding, the body weights of mice that were fed *E. coli* containing the *3D8tscFv* gene were higher than those of the mice that were fed *E. coli* lacking the *3D8scFv* gene, but no significant differences in body weight were observed after 24 h (Fig. 3B). In addition, at the end of the observation period, no remarkable mortality or individual clinical signs were noted in any of the animals (Supplementary Data 1). To detect *E. coli* containing the *3D8scFv* gene in the cultured cells and in the stools of the treated animals, we designed a set of *3D8scFv* gene-specific primers. When tested in the polymerase chain reaction (PCR), this primer set did not result in any nonspecific gene amplification. The predicted size of the fragment amplified by this primer set is 523 bp. *E. coli* 3D8scFv and host *E. coli* cells were used in PCR as positive and negative control templates, respectively. We used two different methods to prepare the PCR templates. In one method, total DNA was extracted directly from the stools of the treated animals; in the other method, total DNA was extracted from cells that had been obtained from stool suspensions and cultured on plates. Unfortunately, we were unable to detect amplified bands from total DNA extracted directly from the stools at any of the time intervals tested (Supplementary Fig. 1). This lack of detection may have been caused by the recovery of an amount of DNA that was smaller than the minimum amount required to serve as a template in PCR amplification. We were also unable to obtain PCR-amplified bands when SD rats were used instead of mice (data not shown). To increase the efficiency of gene

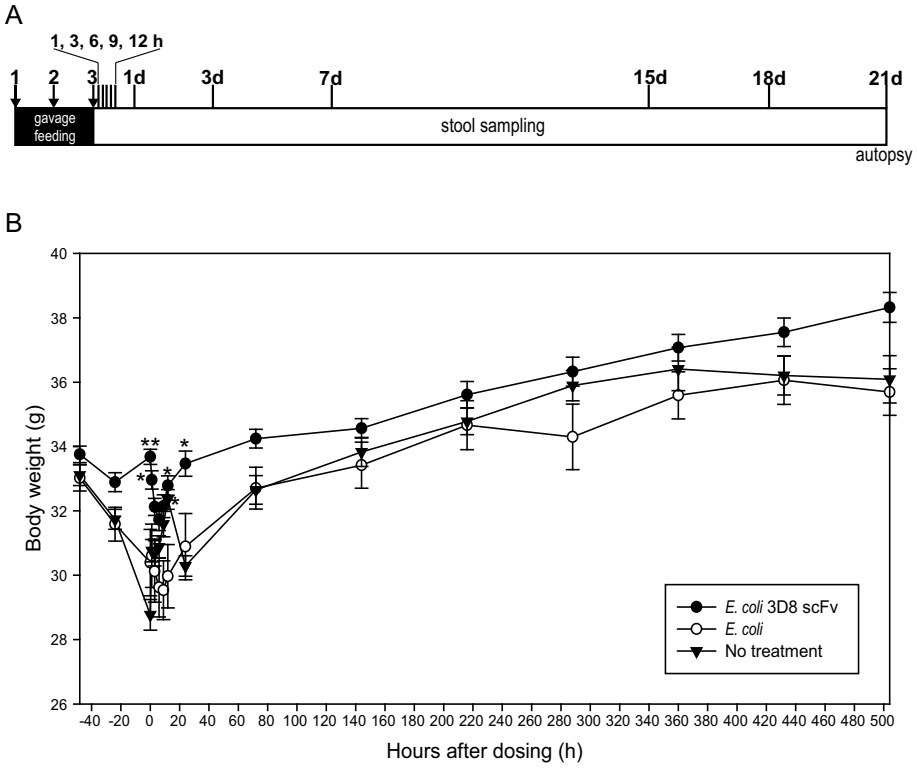

Fig. 3. Schematic plan of the mouse feeding experiments and body weights of the mice. (A) Schematic plan of mouse feeding experiments. Stools were collected 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d after the last feeding of cells or saline. After 21 days, the mice were sacrificed and necropsied. Total DNA was extracted from the stomachs, small intestines, and colons of the animals. **(B)** The body weights of the mice were measured −48 h, −24 h, 0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d after the last feeding of cells or saline.

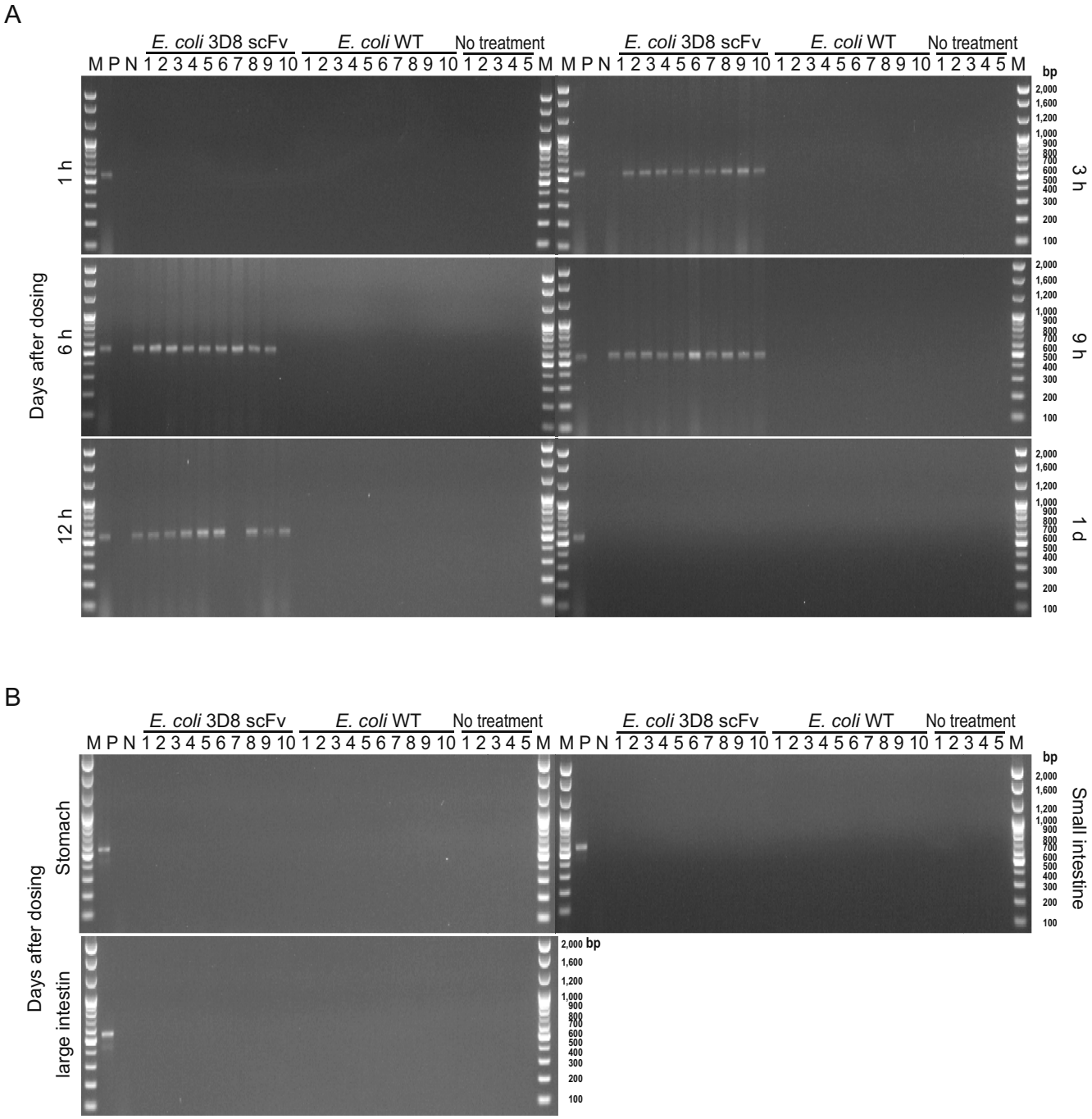

Fig. 4. Detection of *E. coli* 3D8scFv in cultured cells isolated from stools. PCR was performed to detect *E. coli* 3D8scFv using cultured cells isolated from stools 1 h, 3 h, 6 h, 9 h, 12 h and 1 d after the last feeding of cells or saline (A) or using total DNA extracted from the stomachs or small or large intestines of the animals after necropsy (B) with *3D8scFv* gene-specific primers. M, P, and N indicate the DNA size marker, the positive control using the pET42 *3D8scFv* vector as a template, and no template, respectively.

detection, we dissolved stools from the treated animals in saline and cultured the supernatants of these suspensions on bacterial culture plates containing kanamycin. On the kanamycin-containing plates used to culture cells obtained from mice that were fed host *E. coli* or saline, few colonies were formed, whereas hundreds of colonies appeared on the kanamycin-containing plates used to culture cells obtained from mice that were fed *E. coli* 3D8scFv. We picked all colonies from the plates, dissolved them in water, and used them as templates for PCR. *E. coli* 3D8scFv was detected at the 3 h, 6 h, 9 h and 12 h time intervals in nearly all of the cultures obtained from mice that were fed *E. coli* 3D8scFv (Fig. 4, Table 1 and Supplementary Fig. 2), whereas no *E. coli* 3D8scFv was detected in cultures obtained from the stools of mice that had orally ingested either host *E. coli* or saline. The gastrointestinal transit time in mice has been reported to be between 8 h and 22 h (Padmanabhan et al., 2013). These results show that *E. coli* 3D8scFv was excreted within the normal digestion time by mice that were fed *E. coli* 3D8scFv; thus, *E. coli* 3D8scFv did not colonize the animals' gastrointestinal tracts.

3.4. Measurement of the survival of E. coli 3D8scFv using total DNA extracted from the stomach, small intestine and colon

To confirm that all *E. coli* 3D8scFv had been excreted from the mice, we attempted to detect the *3D8scFv* gene in total DNA extracted from

Table 1
Detection of *E. coli* 3D8scFv in cultured cells isolated from stools.

Time after dosing	Group (Positive/number of animals)		
	Non-Treatment Control (5 total test animals)	Control (10 total test animals)	Treatment (10 total test animals)
1 h	ND	ND	ND
3 h	ND	ND	9/10
6 h	ND	ND	9/10
9 h	ND	ND	10/10
12 h	ND	ND	9/10
1 d	ND	ND	ND
3 d	ND	ND	ND
6 d	ND	ND	ND
9 d	ND	ND	ND
12 d	ND	ND	ND
15 d	ND	ND	ND
18 d	ND	ND	ND
21 d	ND	ND	ND

ND: no detection in all animals.

the stomach, small intestine, and colon. On day 21, all animals were anesthetized with CO_2 and necropsied. During the necropsy, no remarkable findings were noted in any of the animals used in this study (Supplementary Fig. 3). The stomachs, small intestines and colons were removed from the mice, and total DNA was extracted from those tissues. The DNA samples were amplified using *3D8scFv* gene-specific primer sets. The *E. coli 3D8scFv* gene was not detected in the DNA extracted from any of the three tissues. Based on these results, we concluded that *E. coli* 3D8scFv did not remain in the gastrointestinal tracts of the mice. As our other results demonstrated, the mice begin to excrete *E. coli* 3D8scFv 1 h after ingestion, and all of the bacteria were excreted from the animals by 12–24 h after the last feeding.

4. Discussion

Studies of feed additives for livestock have addressed not only the effects of the additives themselves but also their potential to deliver beneficial substances to the gastrointestinal tracts of livestock. In this study, we investigated whether an *E. coli* K-12 strain secreting a recombinant nucleic acid antibody that is known to inhibit the multiplication of MNV is capable of colonizing the intestines of mice. *E. coli* is a bacterium that is present in the lower intestines of humans and animals. The detection of *E. coli* in food is one of the characteristic measures used to detect food contamination, and the presence of *E. coli* in food generally indicates that the food was exposed to the excreta of humans or animals. However, one *E. coli* strain, *E. coli* Nissle 1917, has been used as a probiotic. This probiotic was used as a carrier to deliver oral vaccines that protect the intestine from infection by mucosal and hematogenic pathogens (Buddenborg et al., 2008). Many microbial pathogens are known to be transmitted through the mucosal surfaces of the gastrointestinal tracts of the host.

The *E. coli* K-12 strain is known to have a low colonization efficiency in the human gut in comparison to the *E. coli* Nissle 1917 strain, and this difference is expected because the *E. coli* K-12 genome lacks the virulence factors that are present in *E. coli* Nissle 1917 (Grozdanov et al., 2004). Interestingly, although colonization by *E. coli* K-12 does not occur in the human intestine, colonization of the human intestine by genetically modified *E. coli* K-12 has been reported to occur when the intestinal microbiota become unbalanced due to antibiotic treatment or inflammation (Heimesaat et al., 2013). Therefore, we evaluated the possible use of *E. coli* 3D8 scFv as a probiotic to inhibit MNV infection via the colonization of the intestine and the destruction of the intestinal microbiota in mice. In addition, to test whether *E. coli* 3D8scFv complies with the substantial equivalence concept required for safety assessment, we determined whether ingested *E. coli* 3D8scFv are excreted rapidly without colonizing the intestine when the purified 3D8 scFv protein is used as a feed additive. The results of this study showed that orally ingested *E. coli* 3D8scFv is excreted from mice and that the excretion of the bacteria occurs within the normal digestive transit time of the animals without colonization of the gastrointestinal tract by *E. coli* 3D8scFv. These results suggest that while *E. coli* 3D8scFv cannot be used as a probiotic, *E. coli* 3D8scFv can be used as a source of purified 3D8 scFv for use as a feed additive. The purified 3D8 scFv is therefore suitable as a feed additive according to the concept of substantial equivalence.

Further study is required to determine whether the destruction of the intestinal microbiota by MNV infection increases the ability of *E. coli* 3D8scFv to colonize intestinal epithelial cells. In addition, it will be necessary to study whether the imbalance of the intestine microbiota that is caused by *E. coli* 3D8scFv colonization can be reversed to yield a normal intestinal microbiota. With respect to the use of 3D8 scFv in the vaccination of animals, it is necessary to consider whether humans can be infected with residual norovirus present in immunized livestock that do not show symptoms of norovirus infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2018.02.012.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2018.02.012.

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