Corporation obtaining approval, the name of its representative, and the address of its main office

Name: Zoetis Japan Inc.
Applicant: Jorge Perez-Martinez, President & CEO seal
Address: 3-22-7, Yoyogi, Shibuya-ku, Tokyo

Approved Type 1 Use Regulation

Name of the type of Living Modified Organism:	*aroA* gene deleted avian *E.coli* strain EC34195 (Poulvac *E.coli*) (*Escherichia coli*)
Content of the Type 1 Use of Living Modified Organism:	[1] Transportation and storage (including transportation and storage of animals inoculated with genetically modified active live vaccine) [2] When falling under the category of trail aiming to gather materials related to the results of clinical test (hereinafter referred to as "clinical trial") among the materials that shall be submitted by the provisions of Article 14, Paragraph 3 of the Pharmaceutical Affairs Act (Act No. 145 of 1960; PAA), use in accordance with a notification of clinical trial plan submitted based on Article 80-2, Paragraph 2 of PAA and a trial implementation protocol prepared based on Article 7 of the Ordinance for Good Clinical Practice of New Animal Drugs (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No.75 of 1997) [3] Use in accordance with the approval application based on Article 14, Paragraph 1 of PAA (excluding acts that fall under [4] below) [4] Inoculation (inoculation to chicken) [5] Disposal of instruments and residues after inoculation in accordance with the disposal standards for infectious industrial waste stipulated by Article 12-2 of the Waste Management and Public Cleansing Act (Act No.137 of 1970) [6] Disposal that does not fall under [5] above (including the cases that involve with disposal of animals inoculated with genetically modified active live vaccine) [7] Acts incidental to [1] to [6] above
Method of the Type 1 Use of Living Modified Organism:	—

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effects on Biological Diversity

1 Information concerning the recipient organism or the taxonomic species to which the recipient organism belongs

(1) Taxonomic placement and distribution in the natural environment

[1] *E.coli* (including avian *E.coli*)

Escherichia coli (*E.coli*) is a prokaryote and one of bacterial species classified into the *Escherichia* genus, enterobacteriaceae family, enterobacteriales order, gammaproteobacteria class, proteobacteria phylum, eubacteria kingdom, bacteria domain. *E.coli* is gram-negative bacillus, belonging to facultative anaerobic bacteria, and is enteric bacteria that exist inside the digestive tracts, especially in the colons, of warm-blood animals (birds and mammals) in the natural environment. *E.coli* can be further classified into groups called “strains” depending on the characteristics. Since *E.coli* can live inside the intestines of various animals, there exist numerous kinds of strains. As a result, there is a wide range of serotypes (O antigen: more than 154 types, H antigen: more than 49 types) [Literature 1, 5, 6, 7].

E.coli resides inside the intestines of chickens as normal bacterial flora, and *E.coli* that is pathogenic to chicken is specifically called avian pathogenic *Escherichia coli* (APEC) [Literature 7, 8, 10, 15]. Avian *E.coli* is distributed throughout the world [Literature 10].

[2] Recipient avian *E.coli* strain EC34195

The recipient avian *E.coli* strain EC34195 used as the host of modified *E.coli* is a wild strain isolated in the UK from clinical cases of avian colibacillosis, for which in 1995 the Veterinary Laboratories Agency (Weybridge, UK) determined its serotype (O78:K80), colony morphology (L), capacity of haemagglutination, etc. [Reference Material 1, 10].

(2) History and current status of use etc.

Recipient avian *E.coli* strain EC34195: The abovementioned recipient avian *E.coli* strain EC34195 underwent research and development at the UK Veterinary Laboratories Agency from year 2000 [Reference Material 1], and in January 2007 the Fort Dodge Animal Health in the US obtained an approval as *aroA* gene deleted avian *E.coli* strain EC34195 (Poulvac *E.coli*).

(3) Physiological and ecological (biological) properties

1) Basic properties

[1] *E.coli* (including avian *E.coli*)

E.coli is mobile rod-shaped gram-negative facultative anaerobic bacteria, which agglutinates red blood cells. However, the haemagglutination is inhibited under the presence of mannose for many strains [Literature 6, 7].

A majority of *E.coli* have sex pilus, fimbriae and flagellum [Literature 16]. Known fimbriae

include mannose-binding type I fimbriae, K88 fimbriae, K99 fimbriae, P fimbriae, S fimbriae, type IV fimbriae, and curli fimbriae [Literature 6, 15]. Strains of avian *E.coli* have type I fimbriae at a high rate of 70-100%, and P fimbriae at a rate of about 25% [Literature 15]. Regarding other fimbriae, some avian *E.coli* strains are known to have S fimbriae or type IV fimbriae and a large majority of *E.coli* including avian *E.coli* have curli fimbriae.

E.coli grows in the temperature range of 18-44°C or below, with the optimal growth temperature being 37°C [Literature 7]. They grow well and form colonies on nutrient agar media. Some strains exhibit capacity to absorb Congo red on Congo red-added agar media.

Some strains exhibit adhesiveness to cell lines of HEp-2 cells, HT2916E cells, etc., and to specific organ explants.

Serologic properties:

The serotypes of *E.coli* are classified based on O-polysaccharide antigens arising from cell walls (more than 154 types), K antigen which is morphologically identified as capsule (more than 80 types), and H antigen originating from flagellum (more than 49 types) [Literature 6, 7].

In many cases, O1, O2 and O78 are isolated from avian colibacillosis. However, not a small number of avian *E.coli* have other serotypes or unidentifiable serotype [Literature 8, 9, 10].

Biochemical properties [Literature 6, 7, 10]:

Regular biochemical properties of *E.coli* are oxidase negative and catalase positive, and ferments glucose and other sugars. In IMViC tests, they show positivity to indole production and methyl red reaction, and negativity to Voges-Proskauer (VP) reaction and citrate utilization.

Genetic properties:

Genotype of *E.coli* strains is identified using molecular genetic techniques such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

For the identification using MLST, 54.3% of avian *E.coli* serotypes O78 isolated in Japan is ST23. ST117, ST155, ST369, and ST1645 are also identified [Literature 17].

For techniques other than those above, when identifying avian *E.coli*, genes commonly found in strains are occasionally used as markers of the strain [Reference Material 1; Literature 1, 10].

Susceptible animals, route of infection and mode of infection:

E.coli infect mammals including human and birds in general, and the host range is substantially broad. A certain level of serotype-dependent inclination is identifiable in host animals from which bacteria can be isolated.

E.coli reside inside digestive tracts of mammals and birds, excreted out of the body through feces, and are transmitted mainly through oral infection via contaminated food, water, etc. Transmission can occur through respiratory organs or wounds. For chicken, in addition to the

routes of infection above, vertical transmission to eggs occurs [Literature 10]. The forms of transmission include on egg transmission (the surface of hatching egg is contaminated with feces, and *E.coli* intrude inside through the egg shell) and in egg transmission (ovaries or fallopian tubes of breeding chicken are contaminated with *E.coli*, and *E.coli* enter inside an egg formed through the system).

[2] Recipient avian *E.coli strain* EC34195

Biological properties [Reference Material 1]:

The recipient avian *E.coli* strain EC34195 is mobile rod-shaped gram-negative bacteria, which agglutinates red blood cells. However, the haemagglutination is inhibited under the presence of mannose. Transmission electron microscopy identified the presence of Type I fimbriae, curli fimbriae, and flagellum. The recipient avian *E.coli* strain EC34195 grew on CFA agar media, and formed L-colonies. Colonies of the strain adsorbed Congo red. Additionally, they adhered to HEp-2 cells, HT2916E cells, avian respiratory tract explants, and avian proximal intestinal explants.

Serologic properties [Reference Material 1, 17]:

The serotype of recipient avian *E.coli* strain EC34195 was O78:K80.

Biochemical properties:

Since it is a wild strain of avian *E.coli*, the recipient avian *E.coli* strain EC34195 is likely to exhibit the biochemical properties of *E.coli*.

Genetic properties:

Genetic properties of the recipient avian *E.coli* strain are identified by MLST [Reference Material 17] and by inspection of marker genes by PCR [Reference Material 1].

Susceptible animals, route of infection and mode of infection:

The recipient avian *E.coli* strain EC34195 infects chicken. Since it is a wild strain of avian *E.coli*, the route and mode of infection of the recipient avian *E.coli* strain EC34195 are likely to be the same as those of other avian *E.coli* strains.

The possibility of them infecting humans and other animals cannot be denied taking into account the extremely broad host range of *E.coli*.

2) Conditions of livable and growable (multipliable) environment

[1] *E.coli* (including avian *E.coli*)

E.coli does not select a soil or water environment as its habitat, yet can live in such an environment. The lifetime of *E.coli* in soil and compost varies widely from several days to about

1 year affected by various factors including the temperature, moisture, oxygen concentration, pH, nature of the soil, dissolved organic carbon, and microbial community, but the number of living *E.coli* decreases over time [Literature 18, 33]. The lifetime of *E.coli* in water is strongly affected by the temperature. The lifetime is longer in a low temperature than in a high temperature, and it is verified that they disappear in several weeks in tap water sterilized by high pressure steam, stored water or lake water (8°C or 25°C), and in 234 days in spring water (15°C) [Literature 33]. In potable water disinfected by chlorine, *E.coli* disappears rapidly similar to other gram-negative bacteria.

Inside a poultry house, *E.coli* is considered to live for a long period of time by adhering to feces, bedding, soil, dusts inside the poultry house, down inside incubators, egg shell pieces, etc. [Literature 10]. There is a report stating that *E.coli* lived for a long period of time under a dry condition, yet their number reduced by 84-97% in 7 days when the dust was damped [Literature 7].

[2] Recipient avian *E.coli strain* EC34195

Since it is a wild strain of avian *E.coli*, the conditions of livable and growable (multipliable) environment of the recipient avian *E.coli* strain EC34195 is likely to be the same as those of other *E.coli*.

3) Predaceous or parasitic properties

—

4) Mode of breeding or multiplication

[1] *E.coli* (including avian *E.coli*)

Mode of multiplication:

Bacteria including *E.coli* infinitely proliferate if all the conditions necessary for multiplication are provided. Multiplication requires a source of carbon, a source of nitrogen, inorganic ions and water, and is affected by a wide range of conditions such as the temperature, oxygen concentration, pH, and osmotic pressure [Literature 19]. Being facultative anaerobic, *E.coli* can grow both aerobically and anaerobically. In regard to the temperature condition, *E.coli* can grow in the temperature range of 18-44°C or below [Literature 7].

When aerobically grown in liquid media, the growth curve transitions from the lag phase, through log phase, stationary phase, and to the death phase. Transition from the log phase to the stationary and death phases is affected by the temperature, nutrition, microbial density, etc. In general, it is known that the frequency of cell division of *E.coli* is once per about 20 minutes under optimal conditions.

When grown using agar media, *E.coli* become established by adhering to and multiplying on the surface of agar, forming visually observable colonies.

In vivo kinetics:

E.coli are transmitted through oral infection via contaminated food, water, etc. Transmission can occur through respiratory organs or wounds. *E.coli* mainly reside inside the intestines of mammals and birds, and excreted out of the body mainly through feces. They may also ectopically reside in organs other than intestines, such as biliary tracts, urinary tracts, and respiratory organs.

Regarding avian *E.coli*, there are little cases of the main lesion of avian colibacillosis observed in the intestines. It is considered when transmitted through respiratory organs avian *E.coli* adheres to and multiplies on air sacs or lungs, induces inflammation, and then eventually travels through the blood flow and infects other organs [Literature 7, 8, 9, 10]. In many cases, *E.coli* sepsis of newborn chick is caused by egg transmission.

Horizontal transfer of nucleic acid:

E.coli horizontally transfers genes among *E.coli* strains or with other bacteria through bacterial conjugation, transformation or transduction [Literature 20].

In the nature, cases of nucleic acid of prokaryote like bacteria being introduced into nucleic acid of eukaryote are observed for intracellular symbiotic bacteria such as *Wolbachia pipientis* that resides within the body of arthropods [Literature 34]. However, *E.coli* is not intracellular symbiotic bacteria, and there has been no report stating nucleic acid of *E.coli* being introduced into nucleic acid of infected animal. Additionally, results of lineage analysis mainly on human genes do not indicate the possibility of nucleic acid of bacteria being introduced into nucleic acid of vertebrates [Literature 34, 35]. Therefore, the possibility of nucleic acid of *E.coli* being introduced into nucleic acid of infected animal is extremely low.

[2] Recipient avian *E.coli strain* EC34195

Mode of multiplication and *in vivo* kinetics:

For it being a wild strain of *E.coli*, the recipient avian *E.coli* strain EC34195 exhibits the mode of multiplication and *in vivo* kinetics similar to those of other *E.coli*. For modes of multiplication, multiplication in liquid media and on agar media has been observed. For *in vivo* kinetics, it is confirmed that intragastric administration to newborn SPF chick made the strains to become established on the intestines (caecum), and then further introduced into the liver and spleen where the strains were established and maintained for 5 week or more [Reference Material 1].

Horizontal transfer of nucleic acid:

For it being a wild strain of *E.coli*, the recipient avian *E.coli* strain EC34195 horizontally transfers genes among *E.coli* strains or with other bacteria through bacterial conjugation, transformation or transduction, similar to other *E.coli*. As mentioned above, the possibility of

nucleic acid of *E.coli* being introduced into nucleic acid of infected animal is extremely low.

5) Pathogenicity

[1] Avian *E.coli*

The pathogenicity of avian *E.coli* is well observed in poultries such as chicken (meat-type chicken and egg-laying hens) and turkey [Literature 7]. Avian *E.coli* reside inside the intestines of chicken. They are normally harmless and do not cause symptoms in healthy chickens. However, when environmental stress is applied or when ectopic infection of APEC occurs through respiratory organs etc., they may cause airsacculitis and sepsis (*E.coli* sepsis) and experimentally infected representative individuals developed pericarditis, perihepatitis, salpingitis, ophthalmitis, etc. In serious cases, the animal died [Literature 7, 8, 9, 10]. Avian *E.coli* has long been considered to exhibit pathogenicity to birds only, but it was reported that avian *E.coli* strains in the lines having K1 antigens (O1:K1, O2:K1, and O18:K1) had no difference from extraintestinal pathogenic *E.coli* (ExPEC) strains separated from human patients of urinary-tract infection or neonatal meningitis in genotypes and contained pathogenic genes, and potentially cause extraintestinal infection to human [Literature 26]. Through comparison of core genome, avian *E.coli* strains with the same serotype and genotype with the recipient avian *E.coli* strain EC34195 are considered differentiable from ExPEC, because they are genetically close to the serotype O78 of enterotoxigenic *E. coli* (ETEC), different in the line from strains with K1 antigen, and loss of pathogenic gene locus and differences in contained pathogenic genes are identified [Literature 27].

The pathogenicity of *E.coli* is involved with adhesion to recipient cell/organ, intrusion, acquisition of iron, resistance to serum (complement), resistance to mycophage, toxin production, etc. [Literature 15], yet the pathogenic factors that characterize avian *E.coli* are yet to be clarified. In general, avian *E.coli* do not produce exotoxins such as heat-labile and heat-stable enterotoxin, and factors considered to have relatively strong association with outbreak of symptoms are adhesion factors, iron acquisition mechanism and resistance to serum [Literature 8].

Factors that promote outbreak of symptoms include an increase in the concentration of harmful gas (e.g., ammonia, carbon dioxide gas) in the atmosphere of poultry house and irritation of respiratory mucous membrane by dust particles floating in contaminated air [Literature 10]. Malnutrition, especially lack of vitamins A and E reduces the resistance to the disease. In general, infection to mycoplasma or virus with relatively low pathogenicity is often associated with infection with various microbial including *E.coli*, and it is said that complex infection escalates the severity of disease in cases of infection with *E.coli*.

[2] Recipient avian *E.coli strain* EC34195

The recipient avian *E.coli* strain EC34195 is a wild strain of avian *E.coli*, and adheres to respiratory tract explants of chick [Reference Material 1 (Figure 5)]. When it was intragastrically administered to chicken, it infected not only the caecum (intestinal tract) but also spleens and liver, which may induce avian colibacillosis, for 5 weeks or more, and thus it has pathogenic factors related to the adhesion onto and intrusion into extraintestinal tissues of chicken. Therefore, similar to general avian *E.coli*, there is a high risk of outbreak of avian colibacillosis when chicken under stress etc. is infected with the recipient avian *E.coli* strain EC34195.

Regarding wild animals, infection to birds may induce symptoms similar to avian colibacillosis. For other animals, there is no report identifying or indicating its pathogenicity.

For humans, there is no report identifying or indicating pathogenicity of the recipient avian *E.coli* strain EC34195.

Antibiotics to which the recipient avian *E.coli* strain EC34195 is susceptible have been identified. Therefore, administration of antibiotics is considered effective as a therapeutic treatment method. Antibiotics to which the recipient avian *E.coli* strain EC34195 is susceptible include gentamicin, tetracycline, ampicillin, amoxicillin, chloramphenicol and streptomycin [Reference Material 1 (Table 8), 18].

6) Productivity of harmful substances

[1] *E.coli* (including avian *E.coli*)

Harmful substances (toxins) *E.coli* produces can be roughly divided into endotoxins (constituent of outer cell membrane of gram-negative bacteria) and enterotoxins (which are secreted to outside the bacterial body) [Literature 3, 4].

Endotoxins are lipopolysaccharides (LPS) which constitute the outer cell membrane of gram-negative bacteria. The polysaccharide part of LPS is O-antigen of gram-negative bacteria. A lipid called lipid A is responsible of a large majority of biological activity (toxicity) of these endotoxins. Among sepsis, those originating from gram-negative bacteria such as *E.coli* exhibit intravascular coagulation syndrome by the act of endotoxin as well as shock symptoms called endotoxin shock [Literature 3, 28]. While the biological activity of endotoxins varies widely, there is not much difference in the variety among strains, and it is considered that symptoms specific to pathogenic bacteria are not caused by endotoxins [Literature 28]. It is considered it has no specific association with the pathogenicity of avian *E.coli* [Literature 7].

Commonly known enterotoxins are heat-labile enterotoxins and heat-stable enterotoxins of ETEC and verotoxin (Shiga-like toxin) of enterohemorrhagic *E. coli* (EHEC) [Literature 4]. These enterotoxins produced by *E.coli* may induce enteric infection (with the main symptom being minor to serious diarrhea) to mammals such as livestock (e.g., pig, cow) and humans. Carriers of *E.coli* that produce these enterotoxins or have enterotoxin genes are wild birds, pigs, and ruminants (e.g., cow) [Literature 22, 23, 24, 25], and chicken are not generally considered as the only carriers. While there is a report indicating the existence of avian *E.coli* strains that produce cytotoxic enterotoxins, enterotoxins specific to the pathogenicity of avian *E.coli* are not

known [Literature 15]. Other than the above, *E.coli* strains that produce colicin (lethal for bacteria) universally exist in the nature.

[2] Recipient avian *E.coli strain* EC34195

For it being *E.coli*, the recipient avian *E.coli* strain EC34195 produces endotoxins. Similar to other pathogenic bacteria, endotoxins of the recipient avian *E.coli* strain EC34195 are considered not to have association with the pathogenicity of the bacterial strain.

Regarding the production of enterotoxins by the recipient avian *E.coli* strain EC34195, there is no report indicating its production except for the production of colicin [Reference Material 1].

7) Other information

In general avian *E.coli* are weak against heat. Heating destroys the hydrogen bond of constituent proteins of bacteria, leading to protein denaturation and death. Enterotoxins also lose their activities by heating. *E.coli* are weak against disinfectants as well; a majority of disinfectants such as ethanol for disinfection, sodium hypochlorite, povidone iodine, and inverted soap solution (benzalkonium chloride solution) are effective [Literature 2]. However, endotoxin LPS is resistant to heat, chemically stable, and cannot be detoxified even when treated with formalin [Literature 4].

For it being a wild strain of *E.coli*, the recipient avian *E.coli* strain EC34195 exhibits these properties like other avian *E.coli*.

2 Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component element

The procedure of the present genetic recombination is modification using the *aroA* gene (1284 base pairs (bp)) of the recipient avian *E.coli*. In the defective *aroA* gene (*⊿aroA*), the 101-bp domain from the 593rd bp to the 693rd bp of *aroA* gene (a gene that codes 5-enolpyruvylshikimate 3-phosphate synthase, one of enzymes that govern the shikimate pathway for synthesizing aromatic amino acids involved with the growth and metabolism of bacteria) is deleted, and the deleted domain is filled with a GCCCGGGCTAAAGATCTTAAGAATTC sequence (26 bp) from the synthetic oligonucleotide primer used for deleting the gene. This 26-bp sequence contains three restriction enzyme cleavage sequences (*Srf* I, *Bgl* II, *EcoR* I) and two stop codons. As a result, the donor nucleic acid is 1187 bp [1284 bp – 101 bp + 26 bp – 22 bp (5' and 3' terminal parts originating from the *aroA* gene, namely, 5'-atgga and ttagccaggcagcctga-3')].

The entire base sequence of the donor nucleic acid is shown in Figure 1-1, and its restriction map is shown in Figure 1-2. The sequence of the synthetic oligonucleotide primer used for the recombination is shown in Table 1 [Literature 11].

For the donor nucleic acid, in order to delete a part (101 bp, from the 593rd bp to the 693rd bp) of *aroA* gene (1284 bp), synthetic oligonucleotide primers (primer pairs of *aroA*-1/*aroA*-2 and

aroA-3/*aroA*-4 shown in Table 1) were used to partially delete and divide *aroA* gene to produce two PCR amplified products. These were then joined through ligation to prepare the donor nucleic acid (defective *aroA* gene) [Attachment 1 (Figure 3)].

As a step prior to introducing into the testing vector described below, the prepared donor nucleic acid was inserted into the multicloning site of the cloning vector using a commercial TOPO TA cloning vector (Invitrogen: pCR2.1 TOPO; similar to plasmids pUC19 and pBR322 derived from *E.coli* JM109) [Reference Material 2] [Attachment 1 (Figure 4)]. The pCR2.1 TOPO vector was used in the donor nucleic acid preparation process.

Table 1. Synthetic oligonucleotide primer sequence

Primer ID	Primer sequence '5-3'
aroA-1	atccctgacgttacaacc
aroA-2	aaaagatctttagcccgggctagaaaccagatcgcctt
aroA-3	tttagatcttaagaattccagtctccgggtacttat
aroA-4	tccgcgccagctgctcga

□ *Bgl* II restriction sites □ *Srf* I restriction site □ Stop codons □ *EcoR*I restriction site

Figure 1-1. Base sequence of donor nucleic acid (confidencial and nondisclosed)

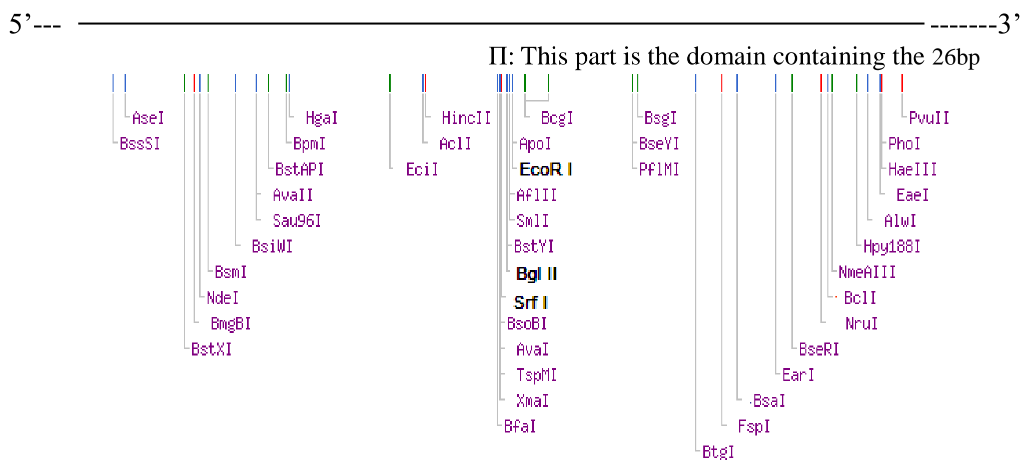

Figure 1-2. Restriction map of donor nucleic acid

(Note: Nomenclaturally, the part of each restriction enzyme name originating from microorganism is written in italic.)

2) Function of component elements

The *aroA* gene deleted avian *E.coli* strain EC34195 lacks a part of *aroA* gene (a gene that codes 5-enolpyruvylshikimate 3-phosphate synthase) and contains a 26-bp synthetic oligonucleotide. The

5-enolpyruvylshikimate 3-phosphate synthase is one of enzymes that govern the shikimate pathway for synthesizing aromatic amino acids [Literature 14]. In general, bacterial strains lacking the function of *aroA* gene require aromatic amino acids (tryptophan, phenylalanine and tyrosine) and aromatic metabolites such as *p*-aminobenzoate (PABA), and their growth and survivability in artificial media and living bodies are restricted [Reference Material 6, 7, 20, 21; Literature 29].

This 26-bp synthetic oligonucleotide contains three restriction enzyme cleavage sequences (*Srf* I, *Bgl* II, *EcoR* I) and two stop codons. The restriction enzyme cleavage sequences are used for distinguishing the strain from other *E.coli* strains. The stop codons play a role to prevent biosynthesis of chimera proteins where the half of the C-terminal side is frameshifted. Homology search [Attachment 7] found no prokaryote gene that has a base sequence completely analogous to the base sequence of this 26-bp synthetic oligonucleotide.

From crystal structure analysis of 5-enolpyruvylshikimate 3-phosphate synthase, it was found that its 15 amino acids that bind to a substrate are widely distributed in the amino acid sequence [Literature 36]. In the case of defective *aroA* gene, due to the presence of stop codons close to the middle of the base sequence, proteins biosynthesized by the defective *aroA* gene are unable to form substrate-binding sites and therefore lose the function as 5-enolpyruvylshikimate 3-phosphate synthase.

(2) Information concerning vectors

1) Name and origin

[1] Testing vector: pKNG101G [Reference Material 4]

This vector originates from pKNG101 plasmid derived from *E.coli* R6K [see 2) [1]-1 pKNG101], which was produced by cloning *Not* I restriction enzyme fragment containing a domain that codes gentamicin resistance gene derived from plasmid pBSL141 onto the *Not* I restriction enzyme site of pKNG101. The gentamicin resistance of this vector is used as a marker to select transformed *E.coli*.

2) Properties

[1] Testing vector: pKNG101G

The testing vector pKNG101G has a base sequence of 7911 bp, where the abovementioned base sequence (925 bp) containing a gentamicin resistance gene was introduced to the *Not* I restriction enzyme cleavage site (gcggccgc) of vector pKNG101 starting at the 2297th bp [Reference Material 3, 4]. The testing vector has both the properties of pKNG101 (see [1]-1 below) and gentamicin resistance as mentioned above, and was used for producing the *aroA* gene deleted strain. In addition, it was confirmed that the gene of this vector never gets introduced into the *aroA* gene deleted avian *E.coli* strain EC34195 in the end [Attachment 1, 3]. This testing vector is not registered in databases open to the public.

The vector "pKNG101" is placed as a material for producing the testing vector "pKNG101G". The

properties of pKNG101 are shown in [1]-1 below.

[1]-1 pKNG101 (plasmid derived from *E.coli* R6K) [Reference Material 3; Literature 12]

It is a suicide vector often used for inserting chromosome of gram-negative bacteria or for inducing chromosomal defects, and has the hereditary components below.

It has a *pir*-R6K replication origin that requires π-protein for replication. It has a *strAB* gene, and is resistant to streptomycin (Sm^R). It has a *sacB* gene that codes levansucrase, and catalyzes hydrolysis of sucrose and synthesis of levan. In plasmid pKNG101, *sacB* gene products induce sucrose sensitivity, and thus the plasmid is identified using sucrose sensitivity as an index. Streptomycin phosphotransferase is used as a selection marker for the presence of this plasmid in bacteria.

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid transferred to the recipient organism

[1] Production of pKNG101G-*ΔaroA* [Reference Material 15]

The entire *aroA* gene of donor nucleic acid was inserted into the pCR2.1 TOPO vector as explained in 2 (1) 1), which was then cleaved using restriction enzymes *Spe* I and *EcoR* V and purified. The product (1239 bp) and the suicide vector (*Sac*B, pKNG101G) predigested with restriction enzyme *Spe* I were both blunted, dephosphorylated, and ligated to produce pKNG101G-*ΔaroA* with a total length of 9154 bp [Figure 2; Attachment 1 (Figure 5)].

[2] Production of *aroA* gene deleted avian *E.coli* strain EC34195

The pKNG101G-*ΔaroA* was introduced into *E.coli* K12 S17 λ*pir* using the electroporation method, resulting in a transformed donor bacterial strain that cannot grow in minimum media [Attachment 1 (Figure 5)].

The donor bacterial strain and the recipient bacterial strain (recipient avian *E.coli* strain EC34195; parent strain) were placed onto nitrocellulose membranes laid on a nutrient agar medium. Strains that underwent bacterial conjugation were selected and single- and double-crossover homologous recombination to produce the modified *E.coli* [Attachment 1 (Figures 6, 7, 8 and 9)].

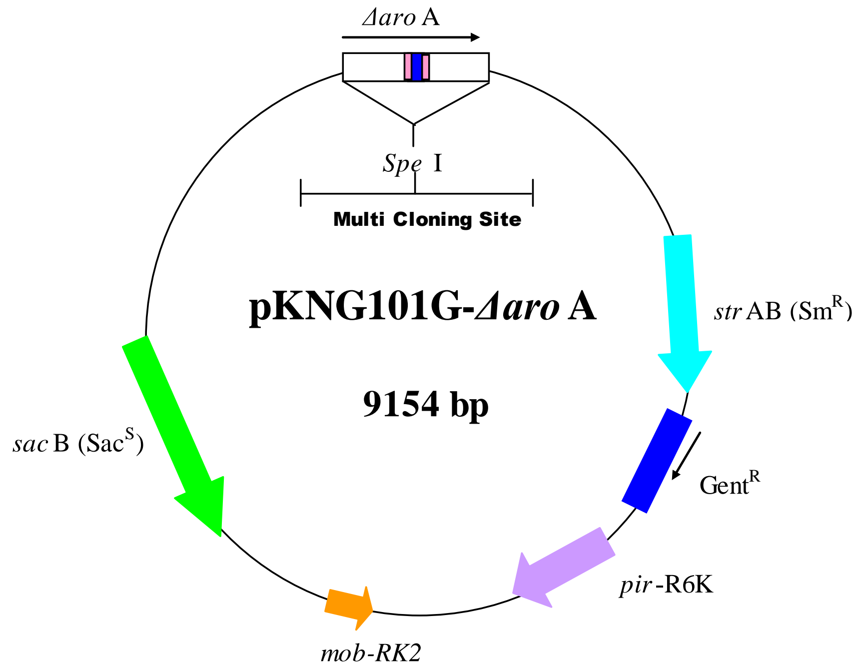

Figure 2. Schematic illustration of pKNG101G-*ΔaroA*

2) Method of transferring nucleic acid transferred to the recipient organism

[1] Method of transferring pKNG101G-*ΔaroA* to *E.coli* K12 S17 λ*pir*

The pKNG101G-*ΔaroA* was introduced into *E.coli* K12 S17 λ*pir* using the electroporation method, resulting in a transformed donor bacterial strain that cannot grow in minimum media [Attachment 1 (Figure 5)].

[2] Method of tansfering defective *aroA* gene to the recipient avian *E.coli* strain EC34195

The pKNG101G-*ΔaroA* was introduced to the recipient avian *E.coli* strain EC34195 through bacterial conjugation with the donor bacterial strain [Attachment 1 (Figure 6)].

After the bacterial conjugation, only the defective *aroA* genes that contain the 26-bp insertion sequence were introduced into the chromosome of recipient avian *E.coli* strain EC34195 through single- and double-crossover homologous recombination.

3) Process of growing living modified organisms

For *E.coli* K12 S17 λ*pir*, donor bacterial strain and recipient bacterial strain used for recombination and production of the defective *aroA* gene, as well as for the master seed and working seed of the *aroA* gene deleted avian *E.coli* strain EC34195, their outlines and growing process are shown below.

1. *E.coli* K12 S17 λ*pir* [Reference Material 5; Literature 13]

It has the transfer genes of broad host IncP type plasmid RP4 introduced into the chromosome of *E.coli*. The *pir* gene is transformed into *E.coli* chromosome by λ phage, and provides π protein

for the suicide vector pKNG101G-*⊿aroA*. It is prepared by culturing on a Luria-Bertani (LB) medium prior to electroporation, and stored on ice until cell fusion.

2. Donor bacterial strain

The donor bacterial strain is transformed K12 S17 λ*pir* containing pKNG101G-*⊿aroA*, and is unable to grow in minimum media. It is prepared by culturing on a gentamicin-added LB medium prior to bacterial conjugation.

3. Recipient bacterial strain

The recipient bacterial strain used was the recipient avian *E.coli* strain EC34195 (parent strain), and is unable to grow in gentamicin-added media. It is prepared by culturing on a gentamicin-free LB medium prior to bacterial conjugation.

E.coli provided for testing was produced simply by recombining the *aroA* gene at the recipient bacterial strain and deleting a part of *aroA* gene (*⊿aroA*) through homologous recombination of the donor bacterial strain (*⊿aroA*) and the recipient bacterial strain (*aroA*) [Attachment 1]. Therefore, there is no possibility of the gene of donor bacterial strain being introduced [Attachment 3].

4. Master seed and working seed of *aroA* gene deleted avian *E.coli* strain EC34195 [Reference Material 14]

The master seed of the *aroA* gene deleted avian *E.coli* strain [Reference Material 14] was provided by the UK Veterinary Laboratories Agency, and the Fort Dodge Animal Health (FDAH) produced the working seed using it.

For production, the maximum allowed number of passage from the master seed to the final product has been specified [Reference Material 14].

(4) Existence state of nucleic acid transferred to cells (inside the recipient organism) and stability of trait expression by the nucleic acid

The defective *aroA* gene containing the transferred 26-bp GCCCGGGCTAAAGATCTTAAGAATTC oligonucleotide exists on the *aroA* locus of the chromosome of the recipient *E.coli* [Attachment 4].

As a result of *in vitro* PCR analysis using the master seed *aroA* gene deleted avian *E.coli* strain EC34195 for creating the vaccine after culturing for 1-5 generations [Reference Material 14], no difference in the size of the amplified gene (1187 bp) was observed among generations, indicating *in vitro* genetic stability [Attachment 5].

Additionally, the *aroA* gene deleted avian *E.coli* strain EC34195 is attenuated by partially deleting the *aroA* gene (that govern the production of aromatic amino acids involved in multiplication of *E.coli*) from the recipient avian *E.coli* strain EC34195 (parent strain), and forced passaging in chicken resulted in recovery by a small fraction in the second generation. At the third generation it became impossible to recover and to transfer, and there was no reversion to virulence observed. As a

result of PCR analysis for *aroA* gene using the *aroA* gene deleted avian *E.coli* strain EC34195 recovered from the second generation, no major difference in the size of the amplified gene was observed compared to that of the master seed without undergoing passaging, indicating the genetic stability of the defective *aroA* gene. From these, it was confirmed that the gene has *in vivo* genetic stability and no reversion to virulence [Attachment 6].

As a side note, the rate of reverse mutation of *E.coli* where the *aroA* gene was deleted using a suicide vector (EC99 strain) was reported to be less than 10^{-12} [Literature 29].

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

The *aroA* gene deleted avian *E.coli* strain EC34195 can be isolated from swab samples collected from internal organs of chickens and those collected from the environment, and can be distinguished from other bacteria by a technique combining isolation culture based on nutritional requirements and biochemical examination using API20E (Sysmex Biomerieux) [Reference Material 19]. This technique has been used in the test reports as attached to the present Assessment Report [Reference Material 6, 7, 20, 21]. The isolated strain can also be identified by obtaining PCR products of *aroA* gene region [Attachment 5; Reference Material 1 (Figure 8), 6 (Figure 1)] or its restriction endonuclease cleavage profile [Reference Material 1 (Figure 8)].

While the transferred oligonucleotide has restriction endonuclease recognition sites *Srf* I and *Bgl* II [Attachment 1], the recipient avian *E.coli* strain EC34195 does not have these restriction endonuclease recognition sites [Attachment 7] and therefore has extremely high specificity.

Additionally, identification can be made by detecting the size of amplified genes through PCR using amplifier primers *aroA*-1 and *aroA*-4. While 1187-bp amplified gene can be found in modified *E.coli*, 1262-bp gene is amplified for the recipient avian *E.coli* strain EC34195 [Attachment 5] and the size difference allows for differentiation.

Regarding the sensitivity of PCR, it is assumed to have the same level of sensitivity as isolation culture, and detection is expected to be possible for several to ten-odd copies. The reliability is assumed to be especially high when combined with the RFLP method.

(6) Difference from the recipient organism or the taxonomic species to which the recipient organism belongs

1) Difference in the properties between the living modified organism and the recipient organism used for preparing it or taxonomic species to which the recipient organism belongs

- Mode of multiplication: The *aroA* gene deleted avian *E.coli* strain EC34195 lacks a part of *aroA* gene [Attachment 1; Reference Material 1]. *aroA* gene deleted bacterial strains such as *E.coli* and salmonella are unable to self-produce substances that are necessary for bacteria to grow, such as aromatic amino acids (tryptophan, phenylalanine and tyrosine) and *p*-aminobenzoate (PABA), and they cannot grow unless such nutrients are supplied from the outside [Literature 29]. This

was confirmed by the fact that the *aroA* gene deleted avian *E.coli* strain EC34195 formulates colonies after 24 hours of culturing at 37°C on MacConkey agar media and trypticase soy agar (TSA) media but not on the minimum agar media [Reference Material 19]. As a side note, regarding the multiplication properties of the *aroA* gene deleted avian *E.coli* strain EC34195 in liquid media (LB media), no difference was observed from the recipient avian *E.coli* strain EC34195 [Reference Material 1 (Figure 10)].

It is considered that *aroA* gene deleted bacterial strains are unable to grow inside the body of birds or mammals that do not have a shikimate pathway [Literature 29]. This was confirmed by the fact that when the *aroA* gene deleted avian *E.coli* strain EC34195 was inoculated to chicken or passaged using chicken the number of chicken the strain is isolated from inside the body or the number of live bacteria decreased over time after inoculation, and it eventually became unable to isolate the bacteria [Attachment 6, 8; Reference Material 6, 7, 20, 21].

- Genetic properties: In the *aroA* gene deleted avian *E.coli* strain EC34195, the 101-bp domain from the 593rd bp to the 693rd bp of *aroA* gene is deleted, and the deleted domain is filled with a 26-bp synthetic oligonucleotide during the process of recombination. There has been no report indicating an existence of the defective *aroA* gene sequence in natural *E.coli* or in the recipient avian *E.coli* strain EC34195. The transferred 26-bp synthetic oligonucleotide is not observed in natural *E.coli* or the recipient avian *E.coli* strain EC34195 [Attachment 7].

  As a side note, the genotype of the *aroA* gene deleted avian *E.coli* strain EC34195 is identified by MLST [Reference Material 17].
- Pathogenicity: When inoculated to chicken by spraying or instilling into the eye, the *aroA* gene deleted avian *E.coli* strain EC34195 infects heart and liver, which may induce avian colibacillosis. However, different from the recipient avian *E.coli* strain EC34195, the *aroA* gene deleted strain disappeared from these organs in 7 days after inoculation without exhibiting clinical symptoms attributable to the infection or forming lesions in the infected tissues [Attachment 6, 8; Reference Material 6, 7, 20, 21]. In a multicenter trial conducted in Morocco [Reference Material 24], no adverse events attributable to inoculation of Poulvac *E.coli* was identified, indicative of not exhibiting pathogenicity to chicken even under an outdoor environment which facilitates outbreak of avian colibacillosis compared to indoor experiment. In addition, no pathogenicity was observed even when inoculated to mice and pigs, animals outside the scope of application [Attachment 9, 10], and no adverse events on inoculated chicken, humans or other animals have been reported in countries to which Poulvac *E.coli* is supplied [Attachment 13]. From the above, it is assumable that the pathogenicity of the *aroA* gene deleted avian *E.coli* strain EC34195 is lower than that of the recipient avian *E.coli* strain EC34195.

  From the nutrient requirements of the *aroA* gene deleted avian *E.coli* strain EC34195, the possibility of the strain growing and being retained inside the body of human for a long period of time is low. From the fact that no adverse events on human attributable to the present preparation

have been identified in countries where Poulvac *E.coli* is licensed, the possibility of human exposure inducing safety concerns is low. This is also supported by the fact that no clinical symptoms indicating induction of intestinal or extraintestinal infection by *E.coli* in human were observed in chicken, mice and pigs inoculated with the *aroA* gene deleted avian *E.coli* strain EC34195.

- Drug susceptibility: The drug susceptibility of the *aroA* gene deleted avian *E.coli* strain EC34195 is the same as that of the recipient avian *E.coli* strain EC34195, and susceptible to antibiotics such as gentamicin, streptomycin, tetracycline, ampicillin, amoxicillin and chloramphenicol [Reference Material 18].

- Productivity of harmful substances: The *aroA* gene deleted avian *E.coli* strain EC34195 has deletion in the *aroA* gene, and is capable of producing endotoxins colicin, similar to the recipient avian *E.coli* strain EC34195. However, due to the deletion in the *aroA* gene, it does not grow and is able to survive only for a certain period of time. Therefore, the amount and period of such harmful substances being produced are more limited than these of the recipient avian *E.coli* strain EC34195.

  The amino acid sequence produced by the 26-bp synthetic oligonucleotide domain inserted into the defective *aroA* gene is serine-proline-glycine [Attachment 4]. As a result of literature search on Medline, there was no report found exhibiting or indicating the relevant amino acid sequence becoming an allergen or a mutagen. Additionally, search using PV-Works (in-company global pharmacovigilance search software) found no adverse events attributable to Poulvac *E.coli*, including adverse events that exhibit or indicate the relevant amino acid sequence becoming an allergen or a mutagen [Attachment 13].

- Infectiousness: It is confirmed that the *aroA* gene deleted avian *E.coli* strain EC34195 can cause an infection when inoculated to chicken [Attachment 6, 8; Reference Material 6, 7, 20, 21]. In addition, taking into account the extremely broad host range of *E.coli*, the possibility of the *aroA* gene deleted avian *E.coli* strain EC34195 being infectious to humans and other animal cannot be denied.

  However, the *aroA* gene deleted avian *E.coli* strain EC34195 is unable to self-biosynthesize aromatic metabolites such as aromatic amino acids and PABA necessary for bacteria. Therefore, its capability in growing and living inside the body of animals where such aromatic metabolites are not biosynthesized is lower than that of the parent strain, recipient avian *E.coli* strain EC34195, and the possibility of it establishing as resident bacteria is low. This was confirmed by the fact that the recipient avian *E.coli* strain EC34195 formed colonies in caecum, liver and spleen of inoculated chicken and bacterial isolation was possible for more than 5 weeks after inoculation [Reference Material 1], while bacterial isolation from inoculated chicken was

possible in the case of the *aroA* gene deleted avian *E.coli* strain EC34195 for a maximum of 7 days (air sac, heart and liver) and 28 days (cloaca) after inoculation [Attachment 8; Reference Material 7, 20, 21], which were less than 5 weeks for any organ.

- Possibility of activating endogenous virus and transferring pathogenicity: For the only difference from the recipient avian *E.coli* strain EC34195 pathogenic to chicken being deletion in the *aroA* gene, the *aroA* gene deleted avian *E.coli* strain EC34195 is likely to have pathogenic genes of avian *E.coli*. Pathogenic genes of *E.coli* are horizontally transferred mainly through bacterial conjugation and transduction, and the *aroA* gene deleted avian *E.coli* strain EC34195 has a possibility to transfer the pathogenicity of avian *E.coli* to other *E.coli* strains and bacterial. However, due to the deletion in the *aroA* gene, the *aroA* gene deleted avian *E.coli* strain EC34195 does not multiply, and able to survive only for a certain period of time. For that reason, the chance of the *aroA* gene deleted avian *E.coli* strain EC34195 horizontally transferring pathogenic genes is lower than that of the recipient avian *E.coli* strain EC34195.

  When inoculated to chicken, mice or pigs, the *aroA* gene deleted avian *E.coli* strain EC34195 did not exhibit any clinical symptoms attributable to the bacterial strain. Therefore, the possibility of it having pathogenicity related to colibacillosis is low including intestinal infection and extraintestinal infection, and the possibility of transferring pathogenicity is low.

- Discharge from inoculated animal and cohabitation infection: Regarding discharge routes of the *aroA* gene deleted avian *E.coli* strain EC34195, when inoculated to SPF chick by spraying or instilling into the eye, the *aroA* gene deleted avian *E.coli* strain EC34195 was detected from cloaca for all inoculated chickens, and was not detected from nasal discharge that was considered to be the other discharge route. For that, it was concluded that the discharge route is excretion of feces [Reference Material 21]. The longest period of time of detection from cloaca was 28 days after inoculation.

  Regarding cohabitation infection, when administered to SPF chick by instilling into the eye, bacteria was isolated from the cloaca of cohabitating chicken for up to 11 days after inoculation, confirming cohabitation infection. However, bacteria was not isolated from 14 days after inoculation, and it was confirmed that the disappearance time in cohabitating chicken was shorter than that in inoculated chicken [Reference Material 20].

  Additionally, in the same study, the *aroA* gene deleted avian *E.coli* strain EC34195 was isolated from the breeding environment (bedding, service water and feed) for a maximum of 21 days after inoculation which was the last day of study. However, no infection was identified in cohabitating chicken from 14 days after inoculation. As such, it was confirmed that the risk of cohabitation infection decreases over time even when the *aroA* gene deleted avian *E.coli* strain EC34195 remains in the environment.

  Furthermore, in any of this study and other studies on cohabitation infection, infection to air sac,

heart, or liver which induces risk of avian colibacillosis outbreak was not identified at all for cohabitating chickens [Attachment 6, 8; Reference Material 6, 7, 20].

- Survivability in the nature: To investigate the stability of the *aroA* gene deleted avian *E.coli* strain EC34195 and the wild strain of avian *E.coli* serotype O78 exposed to bedding inside a poultry house, time-dependent transition in the amount of bacteria at room temperature (18°C) was studied, assuming the common breeding environment of chicken. As a result, no bacteria were detected from bedding inoculated with one dose (assumed dose per bird) of the *aroA* gene deleted avian *E.coli* strain EC34195 after 24 hours. Additionally, no bacteria were detected after 48 hours for the wild strain of avian *E.coli* serotype O78 [Reference Material 12].

  To investigate the survivability of the *aroA* gene deleted avian *E.coli* strain EC34195 and the wild strain of avian *E.coli* serotype O78 exposed to service water and environmental water (water from pond), time-dependent transition in the amount of bacteria at the ambient temperature (20°C) was studied. The number of *aroA* gene deleted avian *E.coli* strain EC34195 and recipient avian *E.coli* strain EC34195 reduced to 1/100-1/10 and 1/10 after 7 days, respectively, confirming that the survivability of the *aroA* gene deleted avian *E.coli* strain EC34195 is equivalent or less than that of the wild strain of avian *E.coli* serotype O78 [Reference Material 16].

  For when inoculated to chicken, the time for the *aroA* gene deleted avian *E.coli* strain EC34195 to disappear from the environment simulating a chicken farm was determined to range from 11 days to 42 days, affected by the amount and method of inoculation and complex environmental factors. When chicken was transferred from the farm after receiving spray inoculation at 2.217×10^8 CFU/dose and adequately being dried, the time for the *aroA* gene deleted avian *E.coli* strain EC34195 to disappear from the environment was 11 days after inoculation [Reference Material 7]. This was the shortest period of time among the studies reviewed. The time for the strain to disappear from the environment when transferred to an environment simulating a farm after inoculating by instilling into the eye or when spray inoculated in the environment simulating a farm was 35 days or 42 days after inoculation, respectively [Reference Material 21]. Additionally, comparison among drinking water, feed and bedding found a tendency of the period of bacterial isolation being short for drinking water and long for bedding.

  While the results of studies on the survivability of the *aroA* gene deleted avian *E.coli* strain EC34195 in the nature were as shown above, it has been clarified that its growth and metabolism are different from those of the recipient avian *E.coli* strain EC34195 and it is unable to grow and duplicate without a supply of aromatic amino acids. For that reason, its survivability in the nature (environment or infected animal/plant) is considered equivalent or less than that of the recipient *E.coli*.

- Horizontal transfer of nucleic acid: Different from the recipient avian *E.coli* strain EC34195, the

aroA gene deleted avian *E.coli* strain EC34195 requires aromatic amino acids and PABA, and the number of live bacteria reduces over time within the body of birds or mammals and in the environment. Additionally, it disappears within 42 days after inoculating to chickens under an environment that simulates a farm mainly used for applying live vaccines. Therefore, its chance of horizontally transferring nucleic acids through bacterial conjugation, transduction and transformation is lower than that of wild strains of *E.coli*.

2) Characteristics of living modified organism such as colony formation and chromogenic properties which enable distinguishing from recipient organism

The *aroA* gene deleted avian *E.coli* strain EC34195 forms colonies on MacConkey agar and TSA media after 24 hours of culturing at 37°C, but not on minimum agar [Reference Material 19].

3 Information concerning the use of Living Modified Organisms

Avian colibacillosis is an infectious disease for chicken caused by *E.coli*, and is globally spreading. It is also causing massive damage to chicken, especially to meat-type chicken, in Japan.

The *aroA* gene deleted avian *E.coli* strain EC34195 is used as the active ingredient of live vaccine Poulvac *E.coli* for preventing avian colibacillosis. The dosage and administration of this live vaccine is "Dissolve the dried vaccine by adding an appropriate amount of purified water, dilute it with purified water so that it becomes 0.1-0.5 mL per chicken, and administer it to chicken aged 1 day or older using a hand-held sprayer device". The planned number of live *aroA* gene deleted avian *E.coli* strain EC34195 bacteria contained in this vaccine is no less than 6.5×10^6 CFU per dose. In order to prevent human exposure spray inoculation, it is planned to specify safety precautions such as "Wear personal protective equipment such as protective goggles, mask and gloves while handling the product, and avoid contact with eye, nose, mouth, etc." and "Wash hands well with soap etc. after handling.", and usage precautions such as "Do not administer this product to chicken within 35 days before the commencement of egg laying."

Due to the deletion in the *aroA* gene, the *aroA* gene deleted avian *E.coli* strain EC34195 has different nutrition requirements from normal *E.coli* and is unable to self-biosynthesize nutrients necessary for bacteria such as aromatic amino acids and PABA. Therefore, its pathogenicity, multiplicability, survivability, etc. are substantially reduced in the body of animals or the environment where such nutrients are lacking. Through that, its safety against inoculated chicken is ensured, the risk of discharge, cohabitation infection and spread in the environment is reduced, and thereby the safety of outdoor use is elevated.

Three products have been approved as avian colibacillosis vaccines in Japan, namely, oil adjuvant-added inactivated vaccine (intramuscular administration; main ingredients: F11 recombinant flagellar antigen and Vero-cell toxic antigen), lipid adjuvant-added inactivated vaccine (ophthalmic administration; main ingredient: whole *E.coli* bacterial cell fragmented antigen), and live avian colibacillosis vaccine (spray administration for the first application, and spray or sprinkle administration for the second application;

main ingredient: *crp* gene deleted *E.coli* serotype O78 that codes cAMP receptor). The present preparation applying for approval is a product similar to the live avian colibacillosis vaccine where *crp* gene deleted *E.coli* serotype O78 is the main agent among these approved preparations, but is differentiated from it for the capability of being effective by single administration using a sprinkler, further saving the labor on vaccination work.

(1) Content of use

[1] Transportation and storage (including transportation and storage of animals inoculated with genetically modified active live vaccine)

[2] When falling under the category of trail aiming to gather materials related to the results of clinical test (hereinafter referred to as “clinical trial”) among the materials that shall be submitted by the provisions of Article 14, Paragraph 3 of the Pharmaceutical Affairs Act (Act No.145 of 1960; PAA), use in accordance with a notification of clinical trial plan submitted based on Article 80-2, Paragraph 2 of PAA and a trial implementation protocol prepared based on Article 7 of the Ordinance for Good Clinical Practice of New Animal Drugs (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No.75 of 1997)

[3] Use in accordance with the approval application based on Article 14, Paragraph 1 of PAA (excluding acts that fall under [4] below)

[4] Inoculation (inoculation to chicken)

[5] Disposal of instruments and residues after inoculation in accordance with the disposal standards for infectious industrial waste stipulated by Article 12-2 of the Waste Management and Public Cleansing Act (Act No.137 of 1970)

[6] Disposal that does not fall under [5] above (including the cases that involve with disposal of animals inoculated with genetically modified active live vaccine)

[7] Acts incidental to [1] to [6] above

(2) Method of use

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(3) Method of collecting information after commencement of Type 1 Use by the corporation applying for approval

On the use of the vaccine during the clinical trial period, monitoring shall be conducted in accordance with the monitoring plan (attached separately).

(4) Measures to prevent potential Adverse Effects on Biological Diversity

See the attached Emergency Measure Plan.

(5) Results of use in an environment similar to the environment planned for use or Type 1 Use at a

laboratory etc.

See Reference Materials 6 (or Attachment 6), 7 (or Attachment 8), 20 and 21 for the results of inoculation to chicken in laboratories etc., and see Reference Material 24 for the results of multicenter trial conducted in Morocco.

(6) Information on the use overseas

[1] Status of approval and reports on adverse events in overseas

Poulvac *E.coli* (brand name of Poulvac *E.coli* in overseas) was first approved in the U.S. (April 2006), followed by in Thailand (February 2008), Canada (September 2008), Malaysia (August 2009), Philippines (March 2010) and in EU (June 2012). The total supply volume confirmable for the U.S., Thailand, Malaysia and Philippines was 219,170,000 dose, 38,000,000 dose, 33,000,000 dose and 9,884,900 dose, respectively (as of April 2012) [Attachment 12]. For the total supply volume, search using PV-Works (in-company global pharmacovigilance search software) found no adverse events on Poulvac *E.coli*-inoculated chicken, human or other animals reported to date for countries to which the product is supplied [Attachment 13], and search using PV-Works and literature search found no reports indicating Adverse Effects on Biological Diversity associated with the use of this vaccine.

[2] Impact assessment on outdoor use in overseas

In the U.S., for the use of Poulvac *E.coli* in field safety test, susceptible animals, possibility of causing recombination or environmental impacts were evaluated by the Center for Veterinary Biologics, Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA) through its Risk Analysis Process, and field use of this vaccine was approved [Attachment 11].

In Canada, at the time of application for licensing for import and domestic use of this vaccine, environmental impacts of this vaccine were assessed by the Veterinary Biologics Section (VBS) of the Canadian Food Inspection Agency [Reference Material 22]. In its Environmental Risk Assessment, VBS concluded that this vaccine is unlikely to cause any serious adverse events to the environment.

In EU, at the time of application for the European Community marketing authorization of this vaccine, Phase I Environmental Risk Assessment was conducted. It was concluded that, while this vaccine corresponds to live vaccine containing genetically modified organisms (GMO) in EU, the risk for when released to the environment is extremely low or negligible. This is described in the assessment document of this vaccine published by the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency [Reference Material 23].

(7) Information on behavior in the body of inoculated animal

[1] Information on the fate of modified live vaccine in the body of inoculated animal

Regarding the routes of discharge of the *aroA* gene deleted avian *E.coli* strain EC34195 from inoculated chicken, when inoculated to SPF chick through spraying or instilling into the eye, it is discharged from cloaca as feces. It is confirmed that bacterial isolation from cloaca becomes impossible in 21-35 days after inoculation [Reference Material 20, 21].

[2] Information on spread of modified live vaccine into the environment from inoculated animal and its excrements, blood, body fluid, eggs, etc.

From [1], there is a possibility for the *aroA* gene deleted avian *E.coli* strain EC34195 to be released into the environment via chicken meat, eggs and feces for 35 days after inoculating the modified live vaccine to chicken. However, taking into account the statements below, different from the case of the recipient avian *E.coli* strain EC34195, it is considered that spread of *aroA* gene deleted avian *E.coli* strain EC34195 released into the environment is temporary and its spatial expansion is limited and the released *aroA* gene deleted avian *E.coli* strain EC34195 disappears after a certain period of time.

- The fact that the survivability of the *aroA* gene deleted avian *E.coli* strain EC34195 directly inoculated to bedding [Reference Material 12] and water [Reference Material 16] was equivalent or lower than that of wild strain of *E.coli*
- The fact that the *aroA* gene deleted avian *E.coli* strain EC34195 disappeared from bedding, water and feed in 11-42 days after vaccination when the *aroA* gene deleted avian *E.coli* strain EC34195 was inoculated to chicken by spraying or instilling in the eye and grown in an environment simulating a farm [Reference Material 7, 11]
- The fact that a study on cohabitation infection identified that the number of cohabitating chicken infected reduced over time and no cohabitation infection was observed from 14 days after vaccination even when the *aroA* gene deleted avian *E.coli* strain EC34195 was remaining in the environment
- The fact that the *aroA* gene deleted avian *E.coli* strain EC34195 requires amino acids and PABA, the number of live bacteria reduces over time inside the body of animal, and therefore the possibility of bacterial retained inside the body of infected animal for a long period of time and the animal becoming a new source of infection is low

For eggs, because inoculation of the present modified live vaccine to chicken is limited to 35 days or more prior to the commencement of egg laying, the possibility of infection via egg is very low as long as the usage instructions are strictly complied.

As a side note, in general, discharged chicken feces are stored and processed in Japan based on the Act on Livestock Manure. Commonly, chicken feces are either processed to be used as manure (e.g. fermenting) or incinerated.

[3] Information on the possibility of vertical infection of the modified live vaccine in inoculated

animal

Because the longest time for the *aroA* gene deleted avian *E.coli* strain EC34195 to become not isolated from cloaca is 35 days after inoculation [Reference Material 21], there is a possibility for infection via egg to take place during this period of time. However, because inoculation of the present modified live vaccine to chicken is limited to 35 days or more prior to the commencement of egg laying, the possibility of infection via egg is very low as long as the usage instructions are strictly observed.

[4] Information on the possibility of spreading to wild animals and plants

The host range of *E.coli* is extremely broad, and for it being avian *E.coli*, there is a possibility that the *aroA* gene deleted avian *E.coli* strain EC34195 to infect wild animals especially birds. From the fact that the period of time for the *aroA* gene deleted avian *E.coli* strain EC34195 to disappear from the environment is 42 days after inoculation, if wild birds or mammals entered into a relevant chicken farm during this period of time, there is a possibility of *aroA* gene deleted avian *E.coli* strain EC34195 infecting wild animals. However, for the nutrient requirements of the *aroA* gene deleted avian *E.coli* strain EC34195, the possibility of infected wild animals retaining bacteria for a long period of time and discharging the bacteria is low, and therefore the spreading is temporary and its spatial expansion will be limited.

[5] Other necessary information

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II. Item-by-item assessment of Adverse Effects on Biological Diversity

1 Property to reduce other microorganisms (property to reduce other microorganisms due to competition, production of harmful substances, etc.)

(1) Identification of wild animals and plants that have a possibility to be affected

Different from the recipient avian *E.coli* strain EC34195, the *aroA* gene deleted avian *E.coli* strain EC34195 is unable to self-biosynthesize essential nutrients such as aromatic amino acids and PABA which bacteria can biosynthesize, and therefore cannot grow in the body of animal or in the environment and can live only for a certain period of time. Therefore, the competitiveness of the *aroA* gene deleted avian *E.coli* strain EC34195 is lower than that of the recipient avian *E.coli* strain EC34195.

The recipient avian *E.coli* strain EC34195 as the parent strain of the *aroA* gene deleted avian *E.coli* strain EC34195 produces colicin that is lethal to microorganisms. The *aroA* gene manipulated for modification has no direct association with the production of colicin, and the *aroA* gene deleted avian *E.coli* strain EC34195 is likely to have the productivity of colicin similar to the recipient. However, because it does not grow in the body of animal and in the environment and its lifetime is limited, the amount and period of colicin production by the *aroA* gene deleted avian *E.coli* strain EC34195 are more limited than these of the recipient avian *E.coli* strain EC34195, and its impact on microorganisms is lower than that of the recipient avian *E.coli* strain EC34195.

Therefore, no microorganism was identified to have a possibility to be affected arising from the property to reduce other microorganisms.

(2) Assessment of the specific content of adverse effects

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(3) Assessment of the possibility of adverse effects

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(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the property to reduce other microorganisms.

2 Pathogenicity (property to infect wild animals and damage the life or growth of such wild animals)

(1) Identification of wild animals and plants that have a possibility to be affected

Considering the facts that the host range of *E.coli* is extremely broad and the *aroA* gene partially deleted in the *aroA* gene deleted avian *E.coli* strain EC34195 has no association with the function of *E.coli* adhering to and intruding inside the body of animals, the possibility of it infecting wild animals

especially birds cannot be denied. However, different from the recipient avian *E.coli* strain EC34195, the *aroA* gene deleted avian *E.coli* strain EC34195 requires aromatic amino acids and PABA, cannot grow in the body of animal where such nutrients are not biosynthesized and can live only for a certain period of time, and therefore the possibility of the strain retained in the body for a long period of time and the infected animal becoming new source of infection is low. This can be confirmed by the fact that the probability of cohabitation infection reduces over time when chickens inoculated with the *aroA* gene deleted avian *E.coli* strain EC34195 are cohabitated with non-inoculated chickens. From the above, even if wild animals are infected, spreading of the *aroA* gene deleted avian *E.coli* strain EC34195 is expected to be temporary and its spatial expansion is limited, and its spreading capacity is considered lower than the recipient avian *E.coli* strain EC34195.

Regarding the pathogenicity, when inoculated by spraying or instilling in the eye, the *aroA* gene deleted avian *E.coli* strain EC34195 infects heart and liver which may induce avian colibacillosis, however, different from the recipient avian *E.coli* strain EC34195, it disappeared from such organs in 7 days after inoculation at latest, clinical symptoms attributable to infection to the relevant bacterial strain were not observed, and no lesion was formed at the infected tissues. In a multicenter trial conducted in Morocco, no adverse events attributable to inoculation of Poulvac *E.coli* were identified, indicative of not being pathogenic to chicken even under an outdoor environment which facilitates outbreak of avian colibacillosis compared to indoor experiment. In addition, no pathogenicity was observed when inoculated to mice and pigs, animals outside the scope of application, and no adverse events on inoculated chicken or other animals have been reported in countries to which Poulvac *E.coli* is supplied. Taking into account the above, it is assumable that the pathogenicity of the *aroA* gene deleted avian *E.coli* strain EC34195 is lower than that of the recipient avian *E.coli* strain EC34195.

Additionally, for the facts including the possibility of the *aroA* gene deleted avian *E.coli* strain EC34195 being retained inside the body of human for a long period of time being low because of its nutrition requirements and no adverse events on human having been reported in countries to which Poulvac *E.coli* is supplied, the possibility of safety concerns arising due to human exposure is low.

From the above, no wild animal or plant was identified to have a possibility to be affected arising from the pathogenicity.

(2) Assessment of the specific content of adverse effects

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(3) Assessment of the possibility of adverse effects

—

(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on

Biological Diversity in Japan arising from the pathogenicity.

3 Productivity of harmful substances (property to produce substances that may affect the living or growth of wild animals or plants)

(1) Identification of wild animals and plants that have a possibility to be affected

Because the *aroA* gene manipulated for modification has no association with production of endotoxins and enterotoxins, similar to the recipient avian *E.coli* strain EC34195, the *aroA* gene deleted avian *E.coli* strain EC34195 produces colicin that serves as both an endotoxin and an enterotoxin. However, because of the partial deletion in the *aroA* gene, the *aroA* gene deleted avian *E.coli* strain EC34195 does not multiply, and can survive inside the body of living organism and in the environment for a certain period of time. Therefore, the amount and period of such harmful substances being produced are more limited than these of the recipient avian *E.coli* strain EC34195. Additionally, the donor nucleic acid does not express functional proteins. Therefore, its productivity of harmful substances is low compared to the recipient avian *E.coli* strain EC34195.

From the above understanding, no wild animal or plant was identified to have a possibility to be affected arising from the productivity of harmful substances

(2) Assessment of the specific content of adverse effects

—

(3) Assessment of the possibility of adverse effects

—

(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the productivity of harmful substances.

4 Property to horizontally transfer nucleic acids (property to transfer or propagate nucleic acid transferred by technique covered by relevant acts to wild animal/plant or other microorganisms)

(1) Identification of wild animals and plants that have a possibility to be affected

E.coli can infect wild animals that belong to birds and mammals. However, there is no report indicative of the nucleic acids of *E.coli* being introduced into the nucleic acids of bird or mammal. Additionally, results of lineage analysis mainly on human genes do not support the possibility of nucleic acids of bacteria being introduced into nucleic acids of vertebrates.

Regarding horizontal transfer of nucleic acids among microorganisms, *E.coli* has a property to horizontally transfer nucleic acids through bacterial conjugation, transformation, and transduction. Due to the partial deletion in the *aroA* gene, the *aroA* gene deleted avian *E.coli* strain EC34195 does

not multiply, and can survive for a certain period of time and its opportunity to horizontally transfer nucleic acids is reduced compared to the recipient *E.coli*. Additionally, it is confirmed that the donor nucleic acid is stably allocated in the *aroA* gene deleted avian *E.coli* strain EC34195 for the period until it disappears. Therefore, the possibility of the donor nucleic acid being horizontally transferred from the *aroA* gene deleted avian *E.coli* strain EC34195 is low. Furthermore, the donor nucleic acid is located not on the plasmid but on the chromosome, and transposon is not used during the process of producing the *aroA* gene deleted avian *E.coli* strain EC34195. Therefore, the possibility of the donor nucleic acid being horizontally transferred due to bacterial conjugation is further lower. On top of that, even if the donor nucleic acid is horizontally transferred, the donor nucleic acid does not express functional proteins. From the above, no microorganism was identified to have a possibility to be affected due to the property to horizontally transfer nucleic acids.

(2) Assessment of the specific content of adverse effects

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(3) Assessment of the possibility of adverse effects

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(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the property to horizontally transfer nucleic acids.

5 Other properties (properties considered appropriate to undergo Adverse Effects on Biological Diversity, including properties to indirectly affect wild animals or plants by changing the base of ecosystem)

For the present modified *E.coli*, it was judged that there were no properties considered appropriate to undergo Adverse Effects on Biological Diversity other than those mentioned above.

III. Comprehensive Assessment of Adverse Effects on Biological Diversity

Regarding the property to reduce other microorganisms, the *aroA* gene deleted avian *E.coli* strain EC34195 has less competitiveness with other microorganisms compared to the recipient avian *E.coli* strain EC34195, and the amount and period of colicin production are limited. From these, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the property to reduce other microorganisms.

Regarding the pathogenicity, the *aroA* gene deleted avian *E.coli* strain EC34195 has less propagating capacity and pathogenicity compared to the recipient avian *E.coli* strain EC34195. From these, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the pathogenicity.

Regarding the productivity of harmful substances, the *aroA* gene deleted avian *E.coli* strain EC34195 has lesser productivity of harmful substances compared to the recipient avian *E.coli* strain EC34195, and the partially deleted *aroA* gene does not express functional proteins. From these, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the productivity of harmful substances.

Regarding the property to horizontally transfer nucleic acids, the possibility of the donor nucleic acid being introduced into the chromosomes of infected animal is low, the opportunity of the *aroA* gene deleted avian *E.coli* strain EC34195 horizontally transferring nucleic acids to other microorganisms is low compared to the recipient avian *E.coli* strain EC34195, and the donor nucleic acid does not express functional proteins. From these, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan arising from the property to horizontally transfer nucleic acids.

Comprehensively assessing the above, it was judged that the use of Living Modified Organism in accordance with Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan.

Glossary

Abbreviation, term	Official name, English name	Brief description
E.coli	*Escherichia coli*	*Escherichia coli*
APEC	Avian Pathogenic *Escherichia coli*	Avian pathogenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*	Enteropathogenic *Escherichia coli* Bacteria that causes enteritis to children. Induces fever, vomiting, and watery diarrhea. It is considered to cause the disease by initially attaching using bundle forming pili, adhering using intimin and affecting the epithelial cell, and by deforming the microvilli (called A/E adhesion).
EIEC	Enteroinvasive *Escherichia coli*	Enteroinvasive *Escherichia coli* Induces enteritis similar to species of dysentery bacillus. This bacteria intrude into the intestinal mucosa, multiply inside the mucosal epithelial cells, and destroy them. Induces fever, abdominal pain, watery diarrhea as well as hematogenous diarrhea containing leucocytes characteristic to bacillary dysentery or mucous diarrhea. Identifiable by Sereny test, OH serotype, ELISA, intrusion to HEp-2 or HeLa cell, etc.
ETEC	Enterotoxigenic *Escherichia coli*	Enterotoxigenic *Escherichia coli* Produces heat-labile and heat-stable enterotoxins.
EHEC	Enterohemorrhagic *Escherichia coli*	Enterohemorrhagic *Escherichia coli* Also known as Shiga toxin-producing *Escherichia coli* (STEC). Produces Shiga-like toxin, enterohemolysin.
EAggEC	Enteroaggrigative *Escherichia coli*	Enteroaggrigative *Escherichia coli* Refers to a group of bacteria that have a property to adhere to epithelial cell by forming aggregate. Prone to spontaneously agglutinate, and has special fimbria (AAF/I). Produces heat-stable enterotoxin (EAST) different from the toxin of

		ETEC. This bacteria is considered to be causal bacteria of chronic diarrhea for children, and causes watery diarrhea, vomiting, dehydration, and occasionally fever, hemorrhagic stool, etc.
ExPEC	Extraintestinal pathogenic *Escherichia coli*	Extraintestinal pathogenic *Escherichia coli* *E.coli* that induces extraintestinal infection by ectopically (aberrantly) infecting biliary tract, urinary tract, respiratory tract, etc. Induces inflammation of infected tissue, sepsis, etc. Uropathogenic *E.coli* and neonatal-causing Meningitis *E.coli* are included in extraintestinal pathogenic *E.coli*.
UPEC	Uropathogenic *Escherichia coli*	Uropathogenic *Escherichia coli* One of extraintestinal pathogenic *E.coli*. Induces urinary tract infections.
NMEC	Neonatal-causing Meningitis *Escherichia coli*	Neonatal-causing Meningitis *Escherichia coli* One of extraintestinal pathogenic *E.coli*. Induces neonatal meningitis.
SPF	Specific Pathogen Free	Free from specific pathogen
bp	base pair	base pair
PCR	Polymerase Chain Reaction	Polymerase chain reaction
Oligonucleotide	Oligonucleotide	Oligonucleotide is a short sequence of nucleotide (DNA or RNA) with the length of about 20 bp or less. Nucleotide has a property to bind with complimentary nucleotide, and oligonucleotide is used as a probe to detect complementary DNA or RNA. Oligonucleotides complementary to DNA are used as primer of polymerase chain reaction.
Primer	Primer	Primer is a short fragment of nucleic acid that has a role to supply 3'OH for when DNA polymerase synthesizes DNA.
aroA	5-enolpyruvylshikimate-3-phosphate synthetase	A gene that codes one of enzymes that govern the shikimate pathway, biosynthesis pathway involved with the growth and metabolism of bacteria (*aroA*: 5-enolpyruvylshikimate-3-phosphate synthetase)

ΔaroA	Delta-*aroA*	The 101-bp domain from the 593rd bp to the 693rd bp of *aroA* gene is deleted
Aromatic amino acid	Aromatic amino acid	Cyclic unsaturated amino acids represented by benzene are called aromatic compounds. Amino acids that have aromatic rings (i.e. phenylalanine, tyrosine, and tryptophan) are called aromatic amino acids.
Plasmid	Plasmid	Plasmid is a common term for DNA molecules that are duplicated in a cell and distributed to daughter cells. Exists inside the cytoplasm of bacteria or yeast, and autonomously carries out duplication independent of the DNA of chromosome. Generally it takes a cyclic double-chain structure. There exist those that cause bacterial conjugation (e.g., F plasmid), those that introduce resistance to antibiotics to the host, etc.
Competent cell or competent *E.coli*	Competent Cell (*E.coli*)	A cell in a state being able to take a foreign DNA (e.g., plasmid, phage) into it. Usually it refers to *E.coli* whose membrane permeability to DNA was increased by cooling down under the presence of calcium ions.
Homologous recombination	Homologous recombination	Homologous recombination is recombination that takes place between domains of DNA with similar (homologous) base sequence. DNA fragmented by various chemical substances or radiation are mainly repaired by homologous recombination.
Single- and double-crossover homologous recombination		When selecting the target gene-deleted strain, it is difficult to directly select a strain that changed from being resistant to the target gene and chemical (or requires nutrition) to being susceptible to them, and selection is made through one selection step after another. Therefore, single crossover for recombining single strand (first step) and double crossover for recombining double strand (second step) are necessary.

Bacterial conjugation	Bacterial conjugation	Various bacteria are known to partially exchange genes among cells through contacting, which is called bacterial conjugation.
Transduction	Transduction	A phenomenon where a phage with specific mode of multiplication takes the gene of infected bacteria into a particle and introduce it inside the next bacteria to be infected.
Transformation	Transformation	Refers to changing genetic properties by externally introducing DNA. Transformation for *E.coli* includes methods to use electroporation (momentarily forms a hole on a cell by electric pulses) and methods to use competent cell by the calcium chloride method.
MLST	Multilocus Sequence Typing	In identification of *E.coli* by MLST by Wirth *et al.* [Reference Material 30], a sequence of about 400 bases each is determined for 7 housekeeping genes, namely, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*, by multiplying by PCR. Based on the determined base sequence information, analysis is carried out using integrated software. The base sequence information of each gene is placed into database by numbering as allele, based on the difference in base sequence information of strains that were analyzed in the past. A sequence type (ST) is assigned and numbering is made depending on the combination of alleles contained in each strain. Closely related ST is classified as ST complex.

Literatures

1. Ezaki, T.: Classification and Identification of Bacteria, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p133-138, Igaku-Shoin, Tokyo (2002)
2. Sato, S.: Disinfection and Sterilization, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p46-55, Igaku-Shoin, Tokyo (2002)
3. Yokochi, T.: Endotoxins, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p121-125, Igaku-Shoin, Tokyo (2002)
4. Noda, M.: Enterotoxins, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p125-132, Igaku-Shoin, Tokyo (2002)
5. Nakada, T.: Phylum-level classification, Bacteria domain, Taxonomy Table http://www2.tba.t-com.ne.jp/nakada/takashi/taxonomy/taxonomy.html (2011)
6. Honda, T.: Rod-shaped gram-negative facultative anaerobic bacteria, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p172-179, Igaku-Shoin, Tokyo (2002)
7. Gross, W.B: Colibacillosis. pp.138-144. *In*: Disease of Poultry. 9th ed. Iowa State University Press, Ames, Iowa, USA, (1991)
8. Murase, T.: Recent Aspects of Colibacillosis in Chickens, J. Jpn. Soc. Poult. Dis. Vol. 46 Special Issue, 1-4, (2010)
9. Nakamura, K.: Colibacillosis, Disease of Chickens, Japanese Society on Poultry Diseases (ed.), p70-73, Japanese Society of Poultry Diseases, (1995)
10. Hashimoto, K.: Colibacillosis, New Encyclopedia of Avian Diseases, Yasuto Takamatsu (ed.), p78-84, Keiyusha, Tokyo, (1982)
11. Duncan, K., et al.: The complete amino acid sequence of Escherichia coli 5-enolpyruvylshikimate 3-phosphate synthase. FEBS Lett. 170:59-63 (1984).
12. Kaniga, K., et al.: A wide-host-range suicide vector for improving reverse genetics in gram- negative bacteria: inactivation of the blaA gene of Yersinia enterocolitica. Gene 109, 137-141. (1991)
13. Simon, et al.: A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria. Biotechnology 1, 784-791. (1983)
14. Shikimic acid and shikimate pathway: Dictionary of Biochemistry 2nd Edition, Kazutomo Imahori *et al.* (eds.), p587-588, Tokyo Kagaku Dojin, Tokyo, (1990)
15. Dziva, F. and Stevens M.P.: Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. Avian Pathology, 37, 355-366. (2008)
16. 5. Extracellular Projections, Chapter 3 General Bacteriology, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p76, Igaku-Shoin, Tokyo (2002)
17. Ozawa, M. et al.: Molecular Typing of Avian Pathogenic *Escherichia coli* O78 Strains in Japan by Using Multilocus Sequence Typing and Pulsed-Field Gel Electrophoresis. J. Vet. Med. Sci. 72, 1517–1520. (2010)
18. Elsas, J.D. et al.: Survival of Escherichia coli in the environment: fundamental and public health aspects.

The ISME Journal, 5, 173–183. (2011)

19. 7. Multiplication of Bacteria, Chapter 3 General Bacteriology, Standard Textbook of Microbiology, Koichi Yamanishi and Keiichi Hirayama (eds.), 8th Edition, p80, Igaku-Shoin, Tokyo (2002)
20. Juhas, M.: Horizontal gene transfer in human pathogens. Crit. Rev. Microbiol., Early Online, 1–8. (2013)
21. Colibacillosis: Concise Dictionary of Veterinary Medicine, Kyoichi Tanaka *et al.* (eds.), p776, Chikusan Shuppansha, Tokyo, (1989)
22. Fukuyama, M. *et al.*: Study on the Verotoxin-producing *Escherichia coli* – Isolation of the Bacteria from Deer Dung–. J. Jpn. Assoc. Infect. Dis., 73, 1140-1144, (1999)
23. Tsukamoto, T. *et al.*: The Serotype and Shiga Toxin Type of Shiga Toxin-Producing *Escherichia Coli* from Humans and Various Animals, J. Jpn. Assoc. Infect. Dis., 76, 167-173, (2002)
24. Fukuyama, M. *et al.*: Isolation and Serotypes of Veto Toxin-producing *Escherichia coli* (VTEC) from Pigeons and Crows, J. Jpn. Assoc. Infect. Dis., 77, 5-9, (2003)
25. Kobayashi, H. et al.: Prevalence and Characteristics of *eae*- and *stx*-Positive Strains of *Escherichia coli* from Wild Birds in the Immediate Environment of Tokyo Bay. Applied and Environmental Microbiology, 75, 292–295. (2009)
26. Moulin-Schouleur, M., et al.: Extraintestinal Pathogenic *Escherichia coli* Strains of Avian and Human Origin: Link between Phylogenetic Relationships and Common Virulence Patterns. J. Clin. Microbiol., 45, 3366–3376. (2007)
27. Dziva, F., et al.: Sequencing and Functional Annotation of Avian Pathogenic *Escherichia coli* Serogroup O78 Strains Reveal the Evolution of *E. coli* Lineages Pathogenic for Poultry via Distinct Mechanisms. Infection and Immunity, 81, 838-849. (2013)
28. Endotoxin: Dictionary of Immunology, Toshiaki Osawa etc. (eds.), p372, Tokyo Kagaku Dojin, Tokyo, (1993)
29. Kariyawasam, S.: Construction, characterization, and evaluation of the vaccine potential of three genetically defined mutants of avian pathogenic *Escherichia coli*. Avian Disease, 48, 287-99. (2004)
30. Wirth, et al.: Sex and virulence in Escherichia coli: an evolutionary perspective. Molecular Microbiology, 60, 1136–1151. (2006)
31. Palchevskiy, V. and Finkel S.E.: *Escherichia coli* Competence Gene Homologs Are Essential for Competitive Fitness and the Use of DNA as a Nutrient. J. Bacteriol, 188, 3902–3910. (2006)
32. Lorenz, M.G. and Wackernagel, W.: Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev., 58, 563–602. (1994)
33. Chauret, C.: Survival and control of *Escherichia coli* O157:H7 in foods, beverages, soil and water. Virulence, 2, 593-601. (2011)
34. Keeling, P.J. and Palmer, J.D.: Horizontal gene transfer in eukaryotic evolution. Nature Review Genetics, 9, 505-618. (2008)
35. Stanhope, M.J., et al.: Phylogenetic analyses do not support horizontal gene transfers from bacteria to vertebrates. Nature, 411, 940-944. (2001)
36. Priestman, M.A., et al.: Molecular basis for the glyphosate-insensitivity of the reaction of 5-enolpyruvylshikimate 3-phosphate synthase with shikimate. FEBS Letters, 579, 5773-5780. (2005)

Attached Materials

Attachments

Attachment 1. **Process of producing the *aroA* gene deleted avian *E.coli* strain EC34195**

Attachment 2. **Differences between the *aroA* gene and the defective *aroA* gene**

Attachment 3. **Confirmation of gene originating from suicide vector (pKNG101G plasmid)**

Attachment 4. **Genetic analysis of the defective *aroA* gene**

Attachment 5. **Genetic stability of the *aroA* gene deleted avian *E.coli* strain EC34195 (*In vitro*)**

Attachment 6. **Genetic stability of the *aroA* gene deleted avian *E.coli* strain EC34195 (*In vivo*)**

Attachment 7. **Homology search of transferred 26-bp oligonucleotide**

Attachment 8. **Safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 in chicken (spray administration)**

Attachment 9. **Safety of the *aroA* gene deleted avian *E.coli* strain EC34195 in mice**

Attachment 10. **Safety of the *aroA* gene deleted avian *E.coli* strain EC34195 in pigs**

Attachment 11. **Veterinary biologics risk assessment of Poulvac *E. coli***

Attachment 12. **Approval status and sales of Poulvac *E. coli***

Attachment 13. **Reports on side effects of Poulvac *E. coli* outside Japan (by PV-Works)**

Attachment 14. **Safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 in SPF chicken (ophthalmological administration)**

Attachment 15. **Discharge and lifetime of the *aroA* gene deleted avian *E.coli* strain EC34195 in the environment**

Attachment 16. **Multicenter trial of Poulvac *E. coli* in Morocco**

Reference Materials

Reference Material 1 to 24: Confidential and nondisclosed

Attachment 1. Process of producing the *aroA* gene deleted avian *E.coli* strain EC34195

Objective:

The materials used for producing the *aroA* gene deleted avian *E.coli* strain EC34195 and its process are described.

Materials:

1. Donor nucleic acid

The 101-bp domain from the 593rd bp to the 693rd bp was deleted from the *aroA* gene of *E.coli*[1] (Figure 1) [1] to create a defective *aroA* gene (Figure 2), giving the donor nucleic acid. A 26-bp foreign insertion sequence originating from the synthetic oligonucleotide primer used for the process of producing the bacterial strain exists in the deleted region of the defective *aroA* gene.

Figure 1. Entire base sequence of *aroA* gene of *E.coli* and deleted region (confidential and nondisclosed)

Figure 2. Entire base sequence of the defective *aroA* gene (confidential and nondisclosed)

2. Synthetic oligonucleotide primer

The synthetic oligonucleotide primers used for creating the *aroA* gene deleted strain were 4 types from *aroA*-1 to *aroA*-4 as shown in Table 1.

Regarding the base sequence of *aroA*-2 and *aroA*-3, about half of the 3' side is the same as the base sequence in the *aroA* gene. To the remaining 5' side, a 20-bp foreign base sequence containing *Srf* I restriction site, *Bgl* II restriction site and stop codons is introduced for *aroA*-2, and a 18-bp foreign base sequence containing *Eco*RI restriction site, *Bgl* II restriction site and stop codons for *aroA*-3.

Table 1. Synthetic oligonucleotide primers provided for producing the *aroA* gene deleted strain

Primer ID	Primer sequence 5'-3'	Position base pair	Accession No
aroA -1	atccctgacgttacaacc	6-23	X00557
aroA -2	aaaagatctttagcccgggctagaaaccagatcgcctt	575-592	X00557
aroA -3	tttagatcttaagaattccagtctccgggtacttat	694-711	X00557
aroA -4	tccgcgccagctgctcga	1250-1267	X00557

□ *Bgl* II restriction sites □ *Srf* I restriction site □ Stop codons □ *Eco*RI restriction site

[1] A gene that codes 5-enolpyruvylshikimate 3-phosphate synthase, one of enzymes that govern the shikimate pathway for synthesizing aromatic amino acids (tryptophan, tyrosine and phenylalanine) involved with the growth and metabolism of bacteria. 1284 base pairs (bp).

3. Vector (testing plasmid)

pKNG101G (suicide vector) [Reference Material 4]

Produced by cloning *Not* I restriction enzyme fragments containing a domain that codes gentamicin resistance gene derived from plasmid pBSL141 onto the *Not* I restriction site of pKNG101. The sucrose sensitivity, streptomycin resistance and gentamicin resistance of this vector are used as markers to select modified *E.coli*.

pCR2.1 TOPO (plasmid derived from *E.coli* JM109: similar to pUC19, pBR322) [Reference Material 2]

A commercial TOPO TA cloning vector (Invitrogen), which was used for deleting 101 bp from *E.coli* O78 *aroA* gene and cloning PCR products amplified by Taq polymerase. This vector was used in the process for preparing the donor nucleic acid.

pKNG101 (plasmid derived from *E.coli* R6K) [Reference Material 3]

A suicide vector often used for inserting chromosome of gram-negative bacteria or for inducing chromosomal defects, and has the hereditary components below.

It has a *pir*-R6K replication origin that requires π-protein for replication. It has a *strAB* gene, and is resistant to streptomycin (Sm^R). It has a *sacB* gene that codes levansucrase, and catalyzes hydrolysis of sucrose and synthesis of levan. In plasmid pKNG101, *sacB* gene products induce sucrose sensitivity, and thus the plasmid is identified using sucrose sensitivity as an index. Streptomycin phosphotransferase is used as a selection marker for the presence of this plasmid in bacteria. This vector "pKNG101" is placed as a material for producing the testing vector "pKNG101G".

4. *E.coli* provided for creating the *aroA* gene deleted strain

E.coli K12 S17 λ*pir* [Reference Material 5]

It has the transfer genes of broad host IncP type plasmid RP4 introduced into the chromosome of *E.coli*. The *pir* gene is transformed into *E.coli* chromosome by λ phage, and provides π protein for the suicide vector pKNG101G-*⊿aroA*.

Donor bacterial strain and recipient bacterial strain

The donor bacterial strain is transformed K12 S17 λ*pir* containing pKNG101G-*⊿aroA*. The recipient bacterial strain used was the recipient avian *E.coli* strain EC34195 (parent strain), wild avian *E.coli* strain isolated from clinical cases of avian colibacillosis in U.K. These bacterial strains are unable to grow in gentamicin-added media.

Process of producing the *aroA* gene deleted avian *E.coli* strain EC34195:

1. Production of donor nucleic acid (Figure 3)

a) PCR[2] was carried out using primer pairs of *aroA*-1/*aroA*-2 and *aroA*-3/*aroA*-4, and 2 types of DNA fragments with about 600 bp was amplified from the *aroA* gene of the parent strain.

b) The terminals of each DNA fragment were cleaved at *Bgl* II, and electrophoresis was carried out. The band was cut out, and each DNA fragment was purified.

c) The same amount of each DNA fragment cleaved by restriction enzyme was mixed, and fragments were joined by ligation (joined DNA fragment lacks from the 593rd to 693rd base pairs in the *aroA* gene, and contains 26-bp inserted sequence originating from the synthetic oligonucleotide primer).

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[2] Polymerase Chain Reaction.

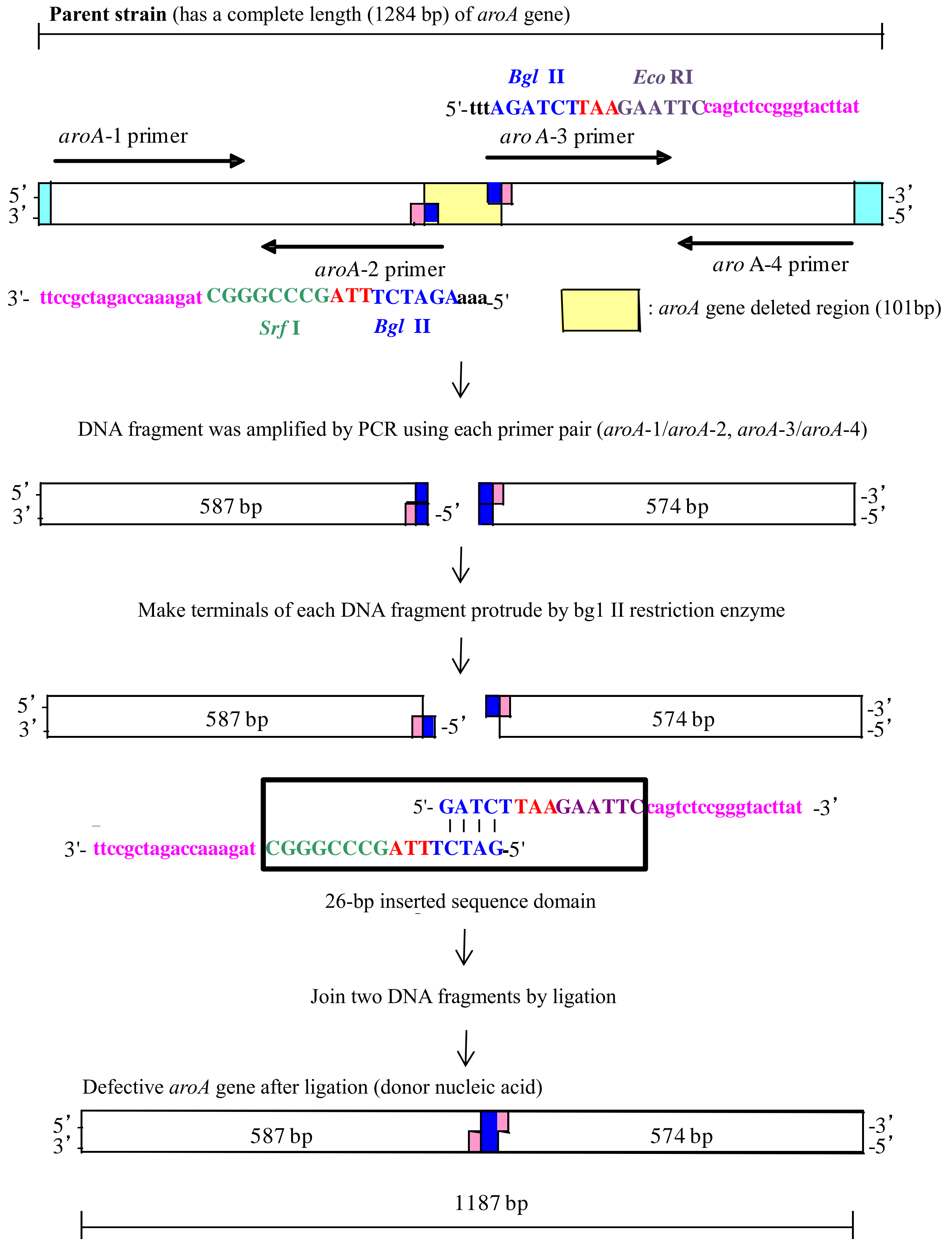

Figure 3. Production of donor nucleic acid

2. Cloning of donor nucleic acid and insertion to suicide vector (pKNG101G) (Figure 4, Figure 5)

a) The donor nucleic acid was inserted into the multicloning site of the plasmid (pCR2.1) vector. The plasmid vector containing the defective *aroA* gene (pCR2.1-*ΔaroA*) was introduced to competent *E.coli*[3] DH5α [2], and successful cloning was confirmed by restriction endonuclease cleavage profile and PCR.

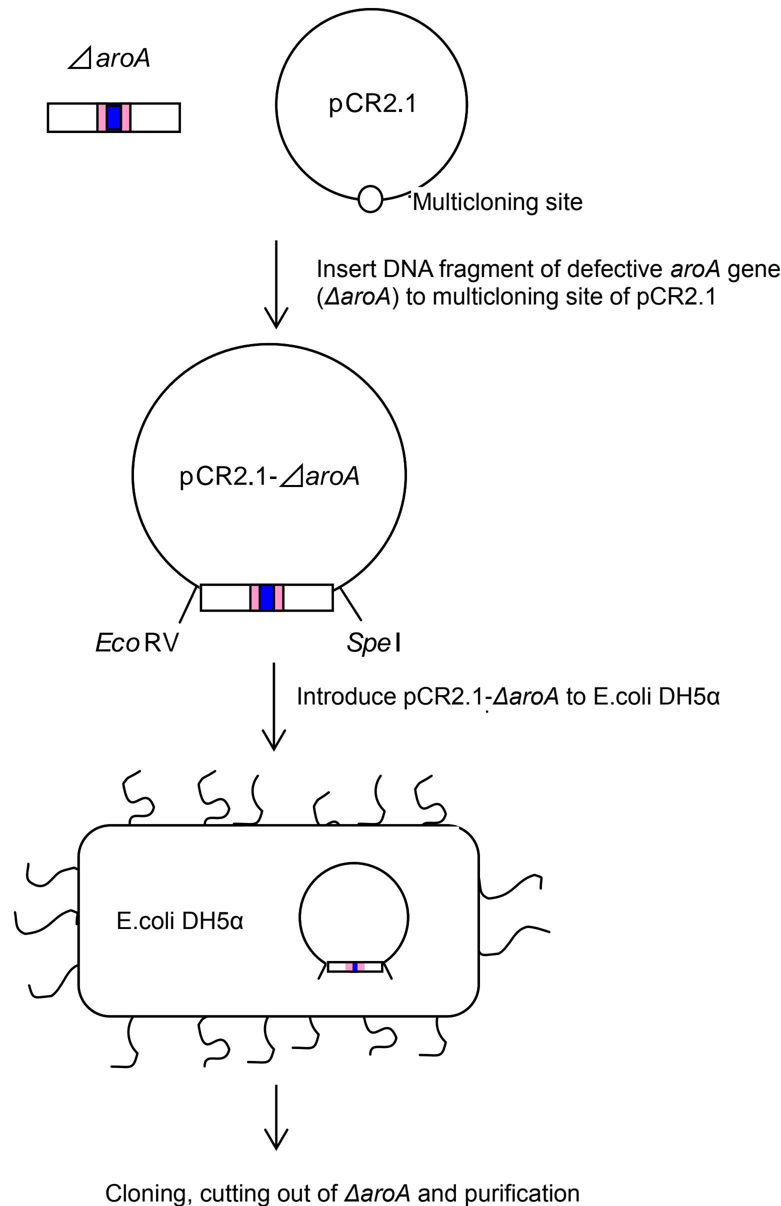

Figure 4. Cloning of donor nucleic acid

b) The entire defective *aroA* gene was cut out from the plasmid (pCR2.1) by making restriction enzymes *Srf* I and *Eco*RV to cleave *Eco*RV and *Spe* I, purified, and inserted into suicide vector (pKNG101G) predigested with restriction enzyme (*Spe* I).

c) The product was introduced by electroporation into *E.coli* K12 S17 λ*pir* that is made electrocompetent[4], and successful cloning was confirmed by restriction endonuclease cleavage profile and PCR.

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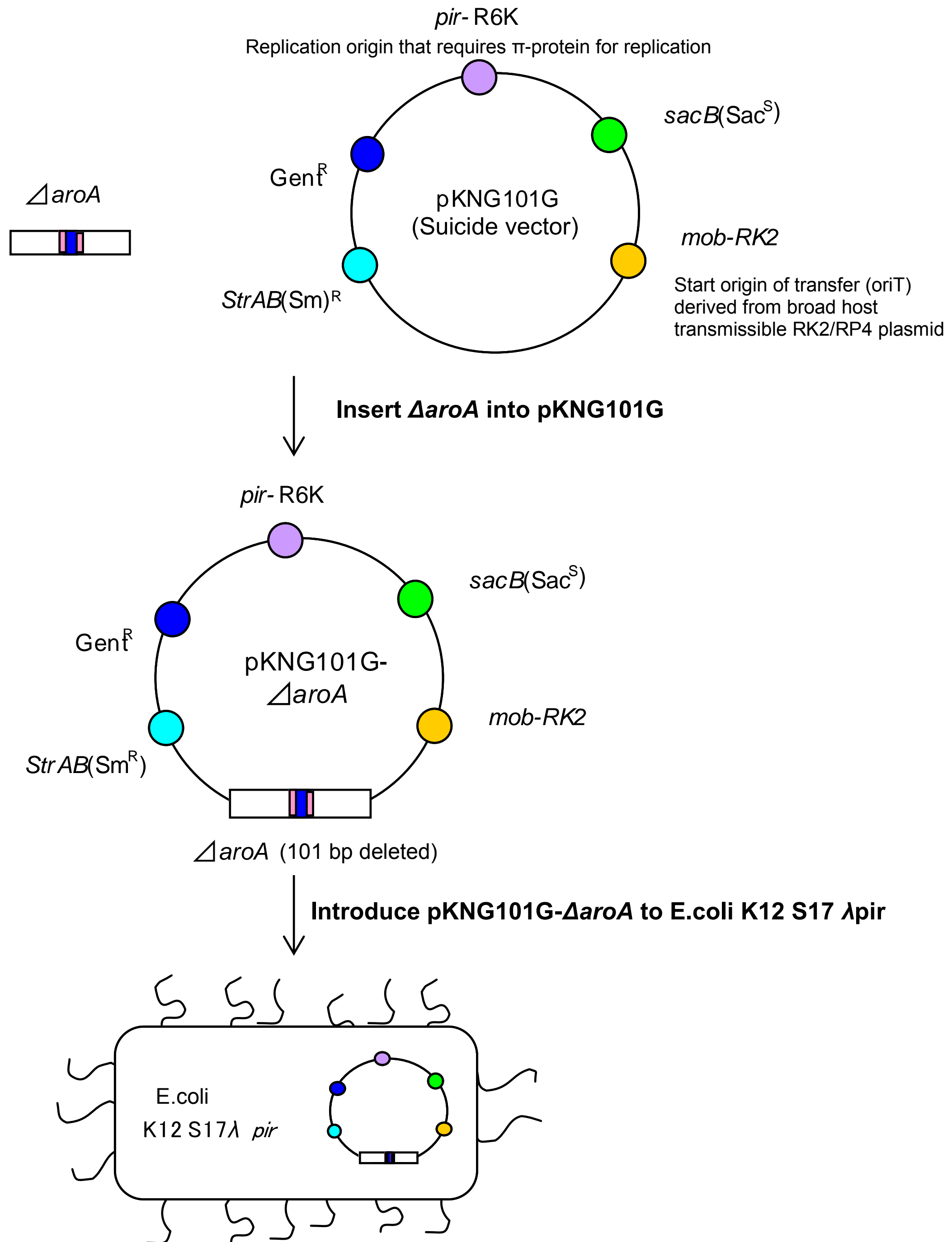
[3] *E.coli* in a state being able to take a foreign DNA (e.g., plasmid, phage DNA) inside the bacterial body. Usually it refers to *E.coli* whose membrane permeability to DNA was increased by cooling down under the presence of calcium ions.

[4] A state made being able to take foreign DNA into the bacterial body for electroporation.



E.coli K12 S17 λpir has pir gene and biosynthesizes π-protein, and is therefore able to replicate pKNG101g-ΔaroA

Figure 5. Insertion of donor nucleic acid (*⊿aroA*) into suicide vector (pKN101G) and insertion of pKNG101G-*⊿aroA* into *E.coli* K12S17*λpir*

3. Conjugation of donor bacterial strain and recipient bacterial strain, and selection of single-crossover product[5] (Figures 6, 7 and 8)

a) Donor bacterial strain and *E.coli* strain EC34195 were conjugated on nitrocellulose membranes laid on a dried nutrient agar.

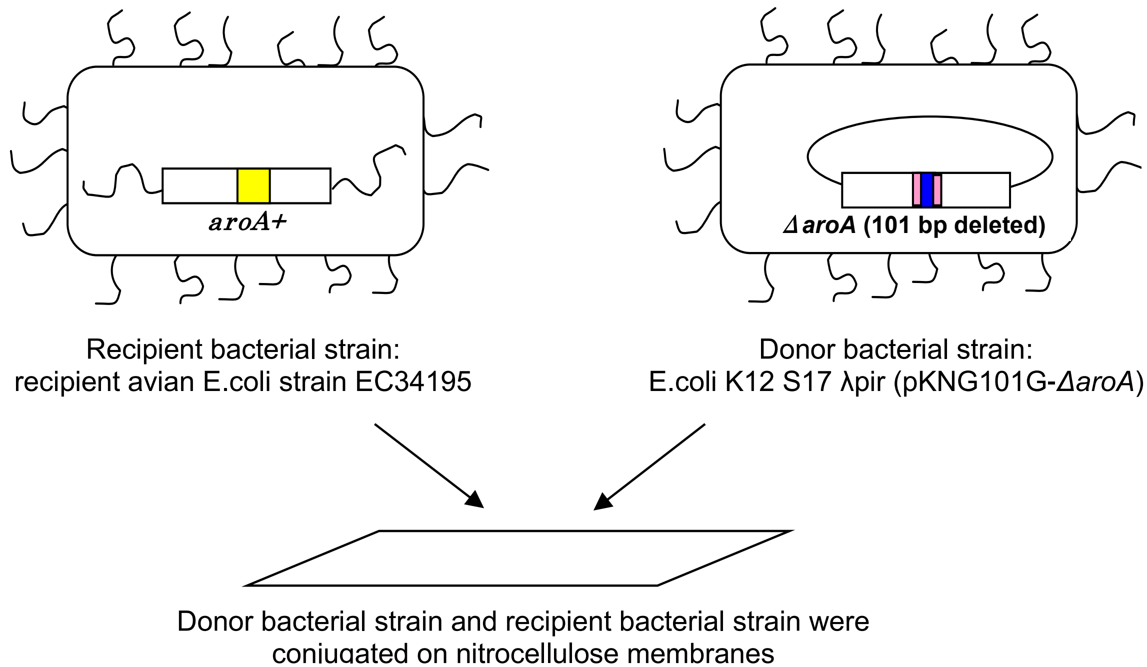


Figure 6. Conjugation of donor bacterial strain and recipient bacterial strain

b) After culturing for 4 hours, the membranes were taken out in a germfree manner, and bacteria attached to the membranes were suspended into an M9 medium.

c) The bacteria suspension was sprinkled onto a glucose-added M9 medium (contains gentamicin and streptomycin), and cultured at 37°C for 48 hours.

d) Developed colonies were successively cultured on a minimum agar medium (contains gentamicin, streptomycin, and aromatic amino acids).

e) Developed colonies were confirmed by PCR. Colonies of mutant (single-crossover product) containing both wild and 101-bp deleted *aroA* genes were selected. The genotype of single-crossover product is shown in Figure 8. Single-crossover product has gentamicin resistance gene, streptomycin resistance gene, sucrose sensitivity gene, defective *aroA* gene (*ΔaroA*), and wild *aroA* gene (*aroA+*).

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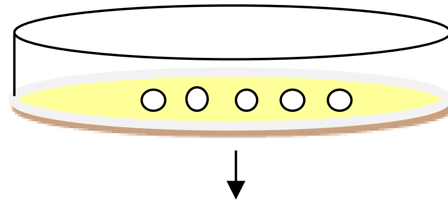
[5] Transgenic organism that underwent homologous recombination by a method where a crossover point is randomly selected and exchange takes place for the section after the crossover point.



Cultured using minimum agar media (contains gentamicin, streptomycin, and aromatic amino acids)

Mutant (single-crossover product) containing both wild and defective *aroA* genes was selected by PCR

Figure 7. Selection of single-crossover product

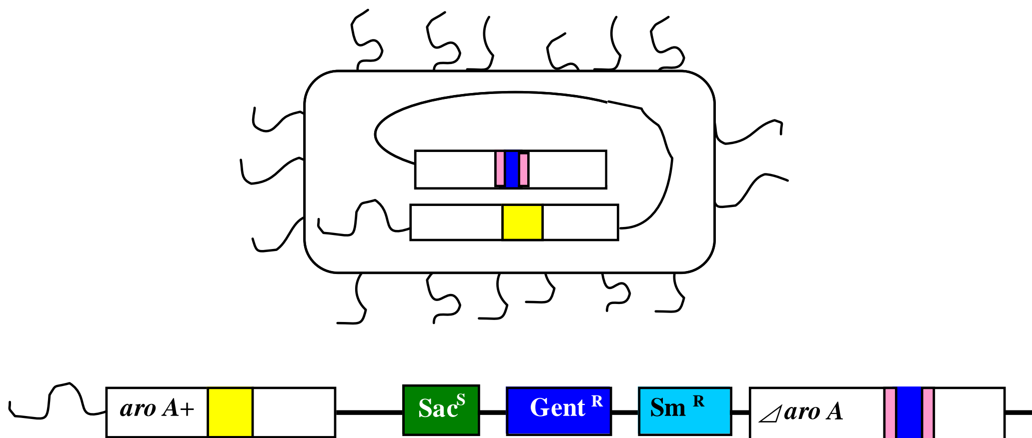


Figure 8. Single-crossover product and its genotype

4. Selection of double-crossover product[6] (Figure 9)

a) The single-crossover product was cultured in a 10% sucrose added LB-G[7] liquid medium (does not contain gentamicin nor streptomycin) at 37°C for 16 hours, with gently shaking. The culture product was diluted serially, sprinkled on a 10% sucrose added LB-G agar medium (does not contain gentamicin nor streptomycin), and cultured at 37°C for 16 hours.

b) Colonies developed on the 10% sucrose added LB-G agar medium was cultured in a LB-G liquid medium, on a gentamicin- and streptomycin-added LB-G agar medium, and on a minimum agar medium, at 37°C for 16 hours. Colonies (double-crossover products) developed only on the LB-G agar medium were passaged on a 5% sheep blood agar medium, and maintained at 4°C.

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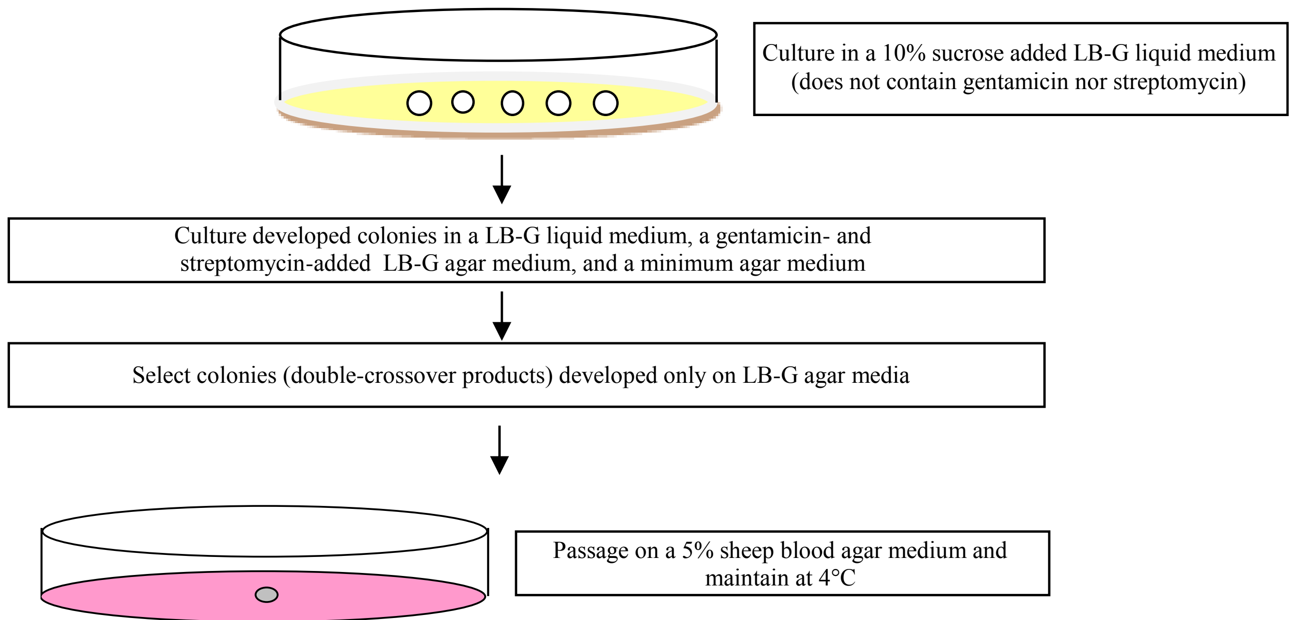
[6] Transgenic organism that underwent homologous recombination by a method where two crossover points were randomly selected and exchange takes place for the section sandwiched by the crossover points. Since it is difficult to directly select from the defective gene, drug resistance or nutrient requirements, the target gene deleted strain was selected step-wise by selecting single-crossover product in the first process and then double-crossover product in the next process.

[7] A Luria-Bertani medium containing glucose. Often used for genetic modification using *E.coli*.

Selective culturing of double-crossover product



Expression type of double-crossover product:
Gentamicin (S) • Streptomycin (S) • Sucrose (R) • *ΔaroA* (101bp–)

Schematic illustration of double-crossover

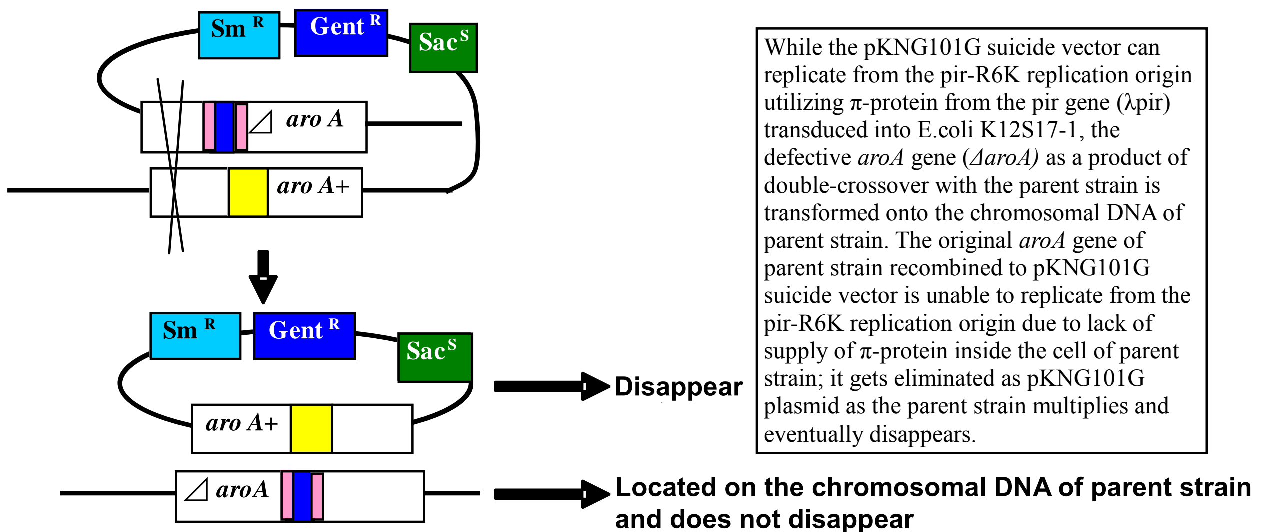


Figure 9. Selective culturing and schematic illustration of double-crossover product

The schematic illustration of the defective *aroA* gene after double-crossover recombination is shown in Figure 10. The total length of this gene inserted at the end was 1209 bp.

Defective *aroA* gene after double-crossover recombination

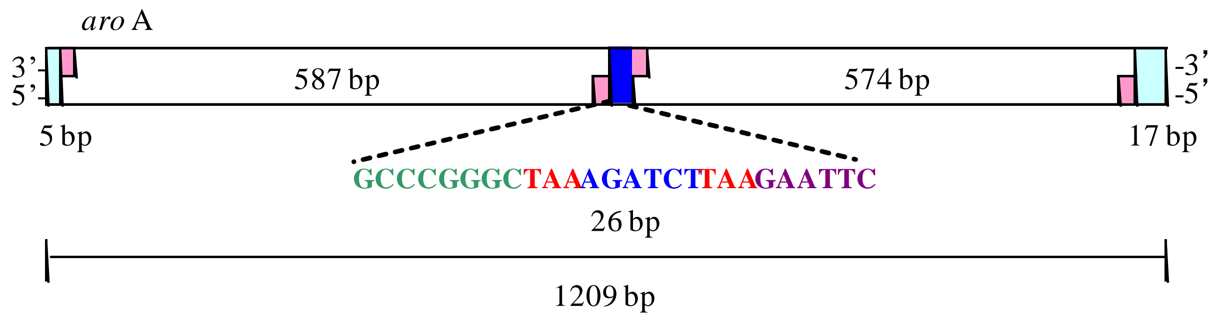


Figure 10. Schematic illustration of defective *aroA* gene after double-crossover recombination

Figure 11. Entire base sequence of *aroA* gene deleted (recombinant) strain (Confidential and nondisclosed)

Literatures:

[1] Duncan, K.A., *et al.*: The complete amino acid sequence of *Escherichia coli* 5-enolpyruvylshikimate 3-phosphate synthase. FEBS Letters, 170, 59-63. (1984)

[2] Woodcock, D.M., *et al.*: Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res., 17, 3469-3478. (1989)

Attachment 2. Differences between the *aroA* gene and the defective *aroA* gene

Objective:

The differences between the *aroA* gene (parent strain) and the defective *aroA* gene (donor nucleic acid and *aroA* deleted avian *E.coli* strain EC34195) are described.

Figure A. Entire base sequence of *aroA* gene of recipient avian *E.coli* strain (parent strain) (Confidential and nondisclosed)

The entire base sequence (1284 bp) of the *aroA* gene of the recipient avian *E.coli* strain EC34195 as the parent strain is shown in Figure A.

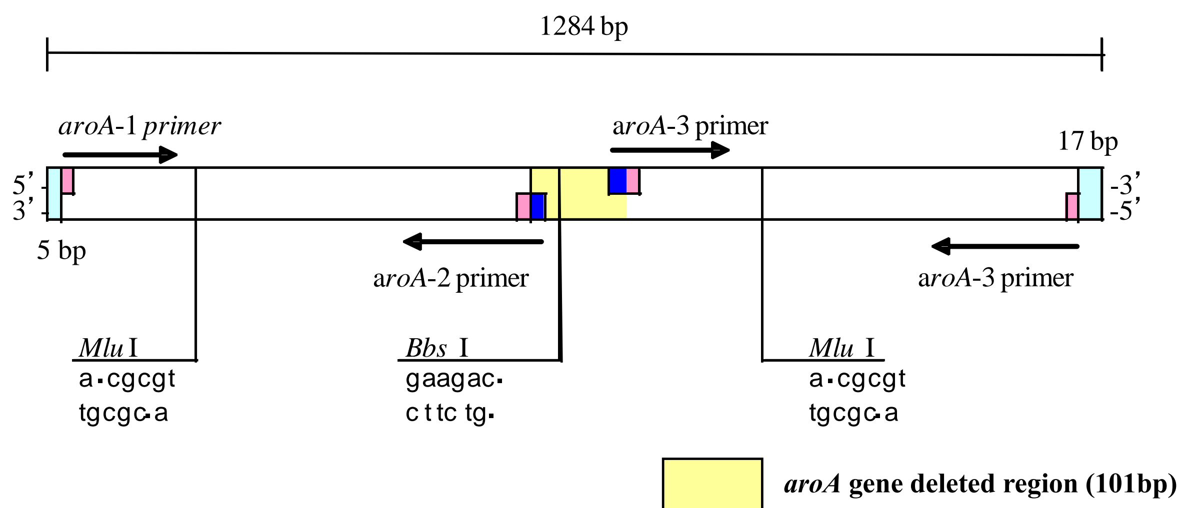


Figure B. Schematic illustration of *aroA* gene of recipient avian E.coli strain EC34195 (parent strain)

The schematic illustration of Figure A is shown in Figure B.

The section shown in yellow in the figure indicates 101-bp base sequence from the 593rd to the 693rd bp that was removed during the process of producing the defective *aroA* gene as the donor nucleic acid.

The sections shown in pink and blue indicate the synthetic oligonucleotide primer [Attachment 1 (Table 1)] regions used during the process of producing the defective *aroA* gene, and the arrows indicate the positional relations of four synthetic oligonucleotide primers and the direction of DNA chain elongation reaction in PCR. The pink sections indicate regions with the same base sequence as the *aroA* gene of parent strain, and the blue sections indicate foreign base sequences originating from synthetic oligonucleotide primers.

The sections shown in light blue indicate 5-bp and 17-bp domains that are not included in the donor nucleic acid (1187 bp).

Figure C. Entire base sequence of defective *aroA* gene of *aroA* gene deleted avian *E.coli* strain EC34195 (Confidential and nondisclosed)

The entire base sequence (1209 bp) of the defective *aroA* gene of the *aroA* gene deleted avian *E.coli* strain EC34195 is shown in Figure C.

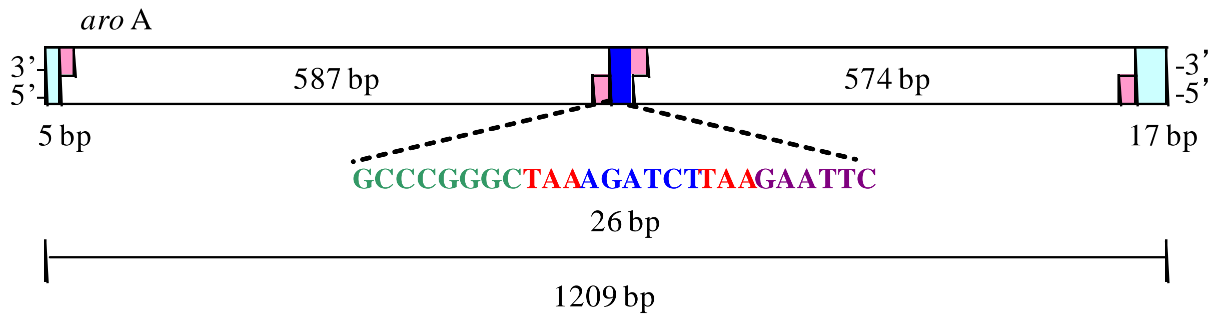


Figure D. Schematic illustration of defective *aroA* gene of *aroA* gene deleted avian *E.coli* strain EC34195

The schematic illustration of Figure C is shown in Figure D.

The sections shown in pink and blue indicate the synthetic oligonucleotide primer [Attachment 1 (Table 1)] domains used during the process of producing the defective *aroA* gene. The pink sections indicate regions with the same base sequence as the *aroA* gene of parent strain, and the blue sections indicates a foreign base sequence **GCCCGGGCTAAAGATCTTAAGAATTC** (26 bp) originating from synthetic oligonucleotide primers.

The sections shown in light blue indicate 5-bp and 17-bp domains that are not included in the donor nucleic acid (1187 bp).

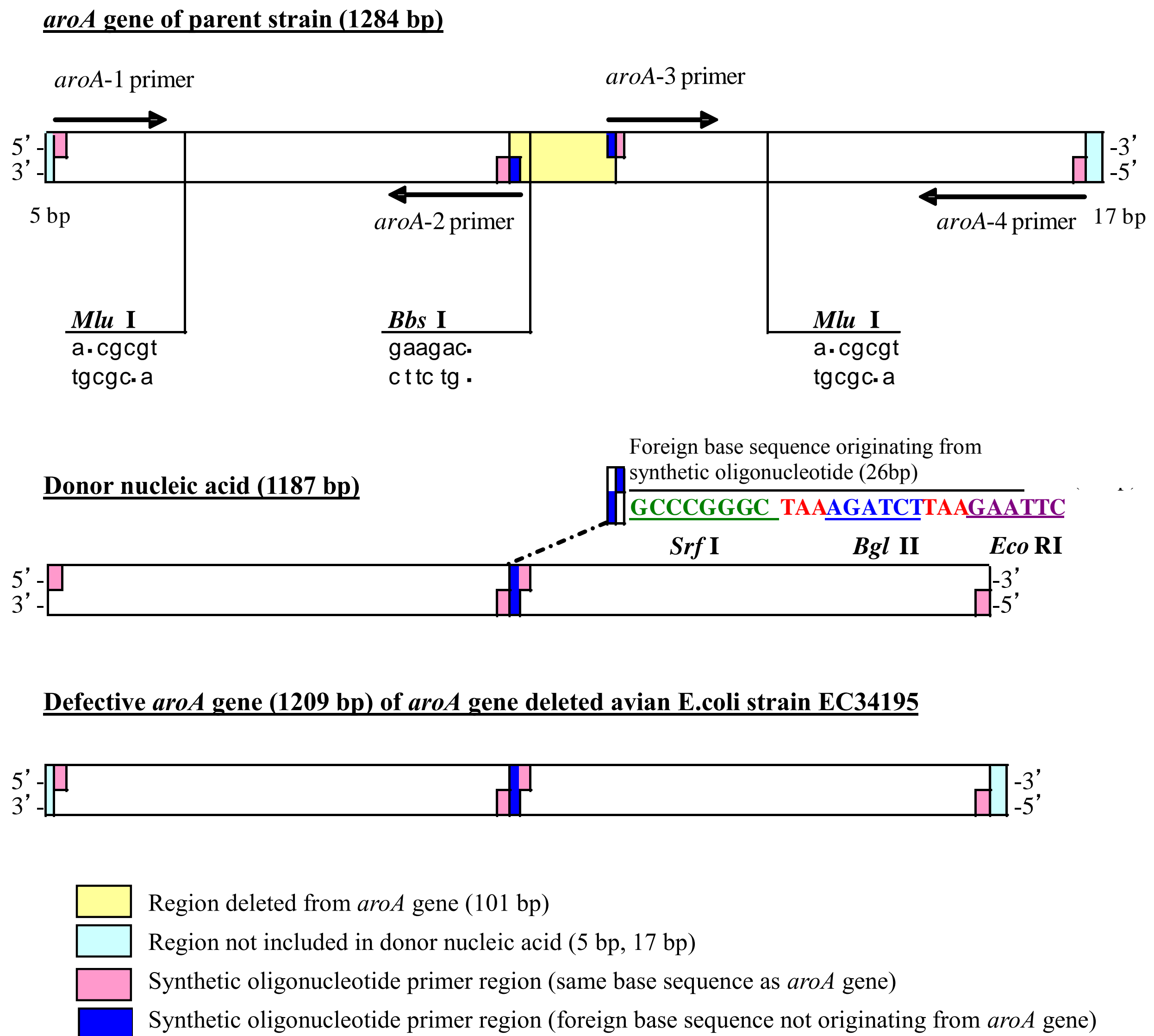


Figure E. Comparison of genes of parent strain, donor nucleic acid, and deleted strain (schematic illustration)

Schematic illustrations of the *aroA* gene (1284 bp) of the parent strain (top), donor nucleic acid (187 bp) (middle), and the defective *aroA* gene of the *aroA* deleted avian *E.coli* strain EC34195 (bottom) are shown in Figure E.

The section shown in yellow in the figure indicates 101-bp base sequence from the 593rd to the 693rd bp that was removed during the process of producing the defective *aroA* gene as the donor nucleic acid.

The sections shown in light blue indicate 5-bp and 17-bp domains that are not included in the donor nucleic acid only.

The sections shown in pink and blue indicate the synthetic oligonucleotide primer [Attachment 1 (Table 1)] regions used during the process of producing the defective *aroA* gene, and the arrows indicate the positional relations of four synthetic oligonucleotide primers and the direction of DNA chain elongation reaction in PCR. The pink sections indicate regions with the same base sequence as the *aroA* gene of parent strain, and the blue sections indicate a foreign base sequence **GCCCGGGCTAAAGATCTTAAGAATTC** (26 bp) originating from synthetic oligonucleotide primers.

Attachment 3. Confirmation of disappearance of genes originating from suicide vector (pKNG101G plasmid)

Objective:

Confirmation was made on absence of the suicide plasmid (pKNK101G plasmid) used during the process of producing the *aroA* gene deleted avian *E.coli* strain EC34195 in the genome of the *aroA* gene deleted avian *E.coli* strain EC34195.

Test method:

The testing bacterial strain were the wild *E.coli* parent strain and *aroA* gene deleted *E.coli* strains including the *aroA* gene deleted avian *E.coli* strain EC34195 (modified *E.coli* O78 strain 17).

After severing with *Bgl II* restriction enzyme, *E.coli* strains and pKNG101G plasmid were subjected to electrophoresis (SDS-PAGE), transcribed to a nylon membrane filer, and underwent Southern hybridization using *SacB* gene coded to pKNG101G plasmid as the probe.

Results and discussions:

No *SacB* gene was detected for wild *E.coli* parent strains including wild *E.coli* O78 parent strain that do not have *SacB* gene (Figure 1: lanes 4, 6 and 8), and no *SacB* gene was detected for modified *E.coli* strains including modified *E.coli* O78 strain 17 (*aroA* gene deleted avian *E.coli* strain EC34195) either (Figure 1: lanes 5, 7 and 9). In contrast to the *E.coli* strains mentioned above, the *SacB* gene was detected in the suicide vector (pKNG101G plasmid) regardless of treatment with *Bgl* II restriction enzyme (Figure 1: lanes 2 and 3). From the above, it was confirmed that the suicide vector disappeared during the process of producing modified *E.coli* strains including the *aroA* gene deleted avian *E.coli* strain EC34195.

A potential reason of the disappearance of the suicide vector was involvement of replication control mechanism of pKNG101G plasmid by π-protein. The pKNG101G plasmid is able to replicate from the *pir*-R6K replication origin under the condition of π-protein being supplied. Normally, *E.coli* does not have *pir* gene that codes π-protein, and therefore it was considered that, after going through transformation of defective *aroA* gene onto the *E.coli* genome by double-crossover recombination, the suicide vector was not replicated and shed from bacteria as the bacteria multiplied, and eventually disappeared.

1: Marker

2: Suicide vector pKNG101G
(not cleaved by restriction enzyme)

3: Suicide vector pKNG101G
(cleaved by *Bgl* II restriction enzyme)

4: Wild *E.coli* O1 parent strain
(cleaved by *Bgl* II restriction enzyme)

5: Modified *E.coli* O1 strain 14
(cleaved by *Bgl* II restriction enzyme)

6: Wild *E.coli* O2 parent strain
(cleaved by *Bgl* II restriction enzyme)

7: Modified *E.coli* O2 strain 15
(cleaved by *Bgl* II restriction enzyme)

8: Wild *E.coli* O78 parent strain
(cleaved by *Bgl* II restriction enzyme)

9: Modified *E.coli* O1 strain 17
(cleaved by *Bgl* II restriction enzyme)
(*aroA* gene deleted avian *E.coli* strain EC34195)

Figure 1. Confirmation of *SacB* gene originating from suicide vector by Southern hybridization (Comparison between parent strains and *aroA* deleted mutants)

Attachment 4. Genetic analysis of the defective *aroA* gene

Objective:

Genetic analysis was carried out for the defective *aroA* gene contained in the *aroA* gene deleted avian *E.coli* strain EC34195.

Method:

Genetic analysis was carried out for defective *aroA* gene, using Vector NTI software (Invitrogen).
Additionally, for open reading frame (ORF) identified by the genetic analysis, homology with base sequence and amino acid sequence of prokaryote genome was searched using BLAST (ver. 2.2.25) of the DNA Data Bank of Japan (DDBJ).

Results:

Genetic analysis using Vector NTI software

1) For the negative chain, a base sequence 70% analogous with T7 promoter Primer (specific RNA polymerase promotor sequence) was found from the 555th base.
2) For the positive chain, two ORFs were identified. One was located from the 1st to 600th base of the defective *aroA* gene, and another from the 709th to 1206th base of the defective *aroA* gene. When the reading frame was shifted, no ORF was identified.
3) For the negative chain, two ORFs were identified. One was located from the 532nd to 855th base of the defective *aroA* gene, and another from the 895th to 1080th of the defective *aroA* gene. When the reading frame was shifted, no ORF was identified.
   (Genetic analysis data in Figures 1 and 2 are confidential and nondisclosed)

Homology search of ORF using BLAST

The assumed genetic products biosynthesized from ORF of the defective *aroA* gene were confirmed to be the first half and second half parts of *aroA* gene products. Among prokaryotes that have *aroA* gene, such as *E.coli*, dysentery bacilli, salmonella, Enterobacter and Citrobacter, high homology was exhibited with *aroA* gene products of *E.coli* and dysentery bacilli. Other than those, there was no analogous base sequence or amino acid sequence identified on the genome of prokaryotes through homology search by BLAST.

Summary and discussions:

The assumed genetic products biosynthesized from two ORFs existing on the defective *aroA* gene exhibited high homology with the first half and second half parts of *aroA* gene products of *E.coli*, dysentery bacilli, etc. Other than those, there was no analogous base sequence or amino acid sequence identified on the genome of prokaryotes.

While the product of *aroA* gene is 5-enolpyruvylshikimate 3-phosphate synthase, it has been clarified that 15 amino acids that bind to a substrate in the relevant enzyme are widely distributed in the amino acid sequence [1]. Therefore, even if the first half and second half parts of 5-enolpyruvylshikimate 3-phosphate synthase were synthesized from the assumed ORFs of the defective *aroA* gene, it is unable to form the substrate-binding site, and will not exert the function as 5-enolpyruvylshikimate 3-phosphate synthase.

From the above, the possibility for the defective *aroA* gene to function as gene of prokaryotes was not identified.

Literature:

[1] Priestman, M.A., *et al.*: Molecular basis for the glyphosate-insensitivity of the reaction of 5-enolpyruvylshikimate 3-phosphate synthase with shikimate. FEBS Letters, 579, 5773-5780. (2005)

Attachment 5. Genetic stability of the *aroA* gene deleted avian *E.coli* strain EC34195 (*In vitro*)

Objective:

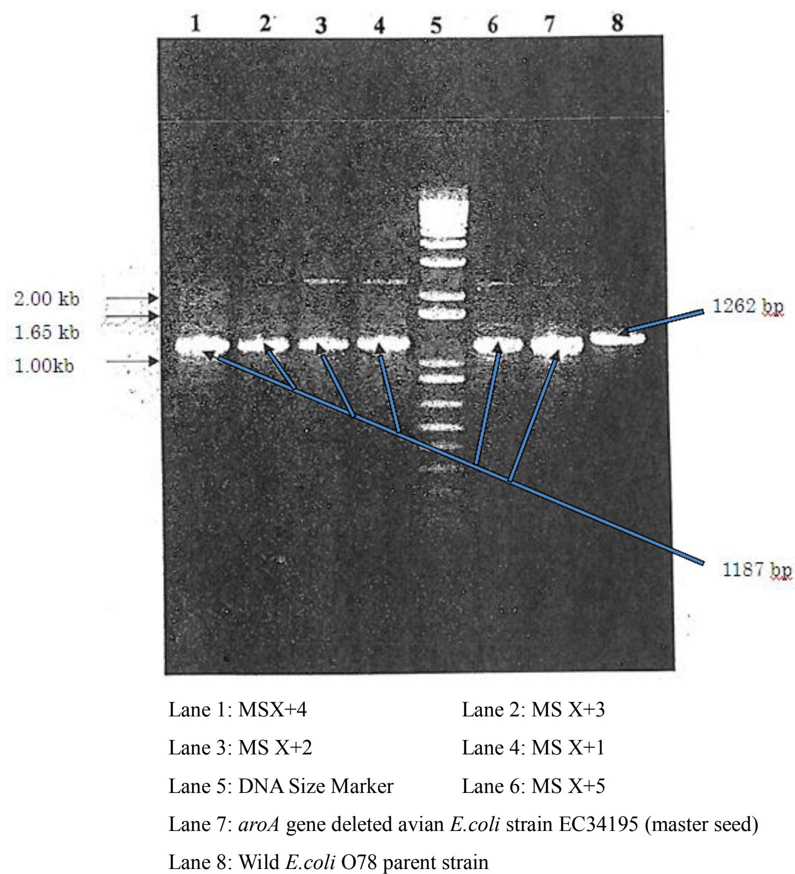
Confirmation was made on the *in vitro* genetic stability of the master seed of *aroA* gene deleted avian *E.coli* strain EC34195 for producing vaccine.

Test method (extract):

For the master seed of *aroA* gene deleted avian *E.coli* strain EC34195 and 1-5 passages of the master seed, *aroA* gene region was detected by polymerase chain reaction (PCR).

Results:

No difference in the size of amplified gene (1187 bp) was observed for the master seed even after passaging for 1-5 generations, confirming *in vitro* genetic stability of the gene.



Lane 1: MSX+4 | Lane 2: MS X+3
Lane 3: MS X+2 | Lane 4: MS X+1
Lane 5: DNA Size Marker | Lane 6: MS X+5
Lane 7: *aroA* gene deleted avian *E.coli* strain EC34195 (master seed)
Lane 8: Wild *E.coli* O78 parent strain

Figure 1. Genetic stability of *aroA* gene deleted avian *E.coli* strain EC34195

Attachment 6. Genetic stability of the *aroA* gene deleted avian *E.coli* strain EC34195 (*In vivo*)

Objective:

In this study, regarding the master seed of *aroA* gene deleted avian *E.coli* strain EC34195 for producing vaccine, successive passage inoculation was carried out using chicken to investigate reversion to virulence.

Test method:

The test design is shown in Table 1. Testing animal used was 1-day old SPF white Leghorn chick (Hy-Vac). Breeding was carried out inside an isolator. The mater seed of the *aroA* gene deleted avian *E.coli* strain EC34195 was prepared with the target value of 5.0 x 10^7 CFU/0.2mL/chick, intratracheally inoculated to 10 testing chicks, which were cohabitated with 5 non-vaccinated chicks. Swab sampling from air sac, heart and liver of each chicken 7 days after inoculation (8-days old), which was recovered to a 50-mL centrifuge tube containing 5 mL of sterile MacConkey bouillon and suspended. The number of *E.coli* in the suspension (unit: CFU/mL) was measured by isolation culture. From the second passage inoculation, the suspension was inoculated for a dose of 0.2 mL/chick, and the chick was bred under similar conditions as the first inoculation.

Identification and confirmation of bacterial strain contained in the recovered suspensions were carried out by polymerase chain reaction (PCR) using 3 types of primers.

Table 1. Test design

No. of passage vaccination	Inoculant*	Route of inoculation*	No. of chicks use for test**	
			Inoculated chick	Cohabitating chick
1st	*aroA* gene deleted avian *E.coli* strain EC34195 (master seed)	Intratracheal	10	5
2nd	Air sac, heart and liver swab suspension	Intratracheal	10	5
3rd	Air sac, heart and liver swab suspension	Intratracheal	10	5

* Inoculation amount is 0.2 mL/chick. Target inoculation amount of the master seed for the first inoculation is 5.0 x 10^7 CFU/0.2mL/chick.

** Among 15 chicks used for the test, 10 chicks were inoculated with the master seed or air sac, heart and liver suspensions, and 5 chicks were made cohabitating without inoculating.

Results:

For the series of passage inoculation, no clinical sign or gross lesion on autopsy was observed for both inoculated chicks and cohabitant chicks.

The *aroA* gene deleted avian *E.coli* strain was recovered from inoculated chicks after the first and second passage inoculation, and not after the third passage inoculation (Table 2).

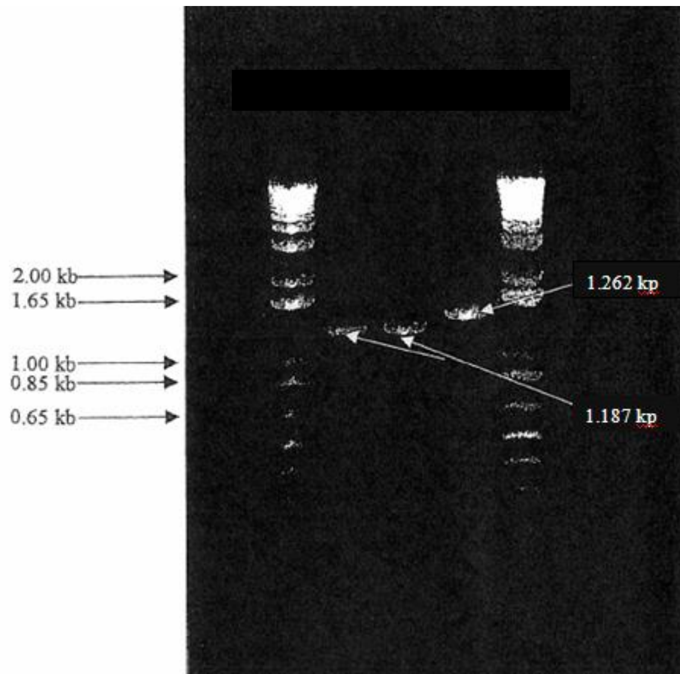
Table 2. Bacterial count of administered inoculant

No. of passage inoculation	Bacterial count of inoculant (CFU*/0.2 mL/chick)	Bacterial count in recovered material (CFU/mL)
1st	1.23×10^7	3.82×10^5
2nd	7.64×10^4	1 colony
3rd	1 colony (unable to measure bacterial count)	–**

* Colony Forming Unit

** Unable to measure

The PCR confirmation test of *E.coli* recovered from the second passage inoculation found that the base sequence of the *aroA* gene region of the second generation isolated strain was shorter than that of wild *E.coli* O78 strain and not different from that of the master seed, confirming that the recovered bacterial strain was the *aroA* gene deleted avian *E.coli* strain EC34195 (Figure 1).



Lane 1: DNA marker

Lane 2: 2nd generation isolated strain #466

Lane 3: *aroA* gene deleted avian E.coli strain EC34195 (master seed)

Lane 4: Wild *E.coli* strain O78

Lane 5: DNA marker

Figure 1. PCR analysis of *aroA* gene for wild E.coli strain and *aroA* gene deleted avian *E.coli* strain EC34195

The recovery rate of the *aroA* gene deleted avian *E.coli* strain was 40% (4/10) from the first passage inoculation, 10% (1/10) from the second passage inoculation, and 0% (0/10) from the third passage inoculation (Table 3). The *aroA* gene deleted avian *E.coli* strain was not recovered from cohabitating chicks for each passage inoculation.

Table 3. Reversion to virulence study: Bacterial isolation data

No. of passage inoculation	Route of inoculation	*E. coli aroA*-*No. of positives/No. of culture	
		Inoculated	Non-inoculated (cohabitating)
1st	Intratracheal	4/10	0/5
2nd	Intratracheal	1/10	0/5
3rd	Intratracheal	0/10	0/5

* *aroA* gene deleted avian *E.coli* strain

Summary and discussions:

No clinical sign or gross lesion on autopsy was observed for inoculated chickens, and when the *aroA* gene deleted avian *E.coli* strain EC34195 was successive passaged using chicks it became impossible to recover and to passage it in the third generation, confirming no observation of reversion to virulence.

Additionally, in the PCR confirmation test of the *aroA* gene deleted avian *E.coli* recovered from the second passage inoculation, it was indicated that the *aroA* gene deleted avian *E.coli* was not different from the master seed of the *aroA* gene deleted avian *E.coli* strain EC34195, confirming *in vivo* genetic stability.

Attachment 7. Homology search of transferred 26-bp oligonucleotide

Objective:

Investigation was carried out on whether the base sequence of 26-bp oligonucleotide inserted into the *aroA* gene deleted avian *E.coli* strain EC34195 was homologous to any base sequence in the genome of *E.coli* or other bacteria.

Test method:

Homology with the inserted 26-bp oligonucleotide was searched using BLASTN 2.2.24 of DNA data bank of Japan (DDBJ) of the National Institute of Genetics, Research Organization of Information and Systems.

Additionally, for the organisms identified by the search, genes with base sequence highly analogous to the inserted 26-bp oligonucleotide were clarified through search using BLAST2.2.26 of the National Center of Biotechnology Information (NCBI).

Results:

Results of homology search

As a result of homology search, homology of 17-19 bp with the inserted 26-bp base sequence was observed for *Lactobacillus amylovorus*, *Staphylococcus aureus*, *Thermodesulfobium narugense*, *Shewanella sp.*, *Clostridiales sp.*, *Vibrio sp.*, *Vibrio parahaemolyticus*, and *Streptococcus uberis*. However, no organism was identified to have homology with the inserted 26-bp base sequence for the entire length (Table 1).

Table 1. Results of homology search

GenBank accession No.	GenBank registration name	Number of homologous bases (out of 26 bp)
CP002609	Lactobacillus amylovorus GRL 1118, complete genome.	19 bp
GQ900418	Staphylococcus aureus plasmid SAP063A, complete sequence.	18 bp
CP002690	Thermodesulfobium narugense DSM 14796, complete genome.	17 bp
CP000446	Shewanella sp. MR-4, complete genome	17 bp
FP929061	Clostridiales sp. SSC/2 draft genome.	17 bp
CP001806	Vibrio sp. Ex25 chromosome 2, complete sequence.	17 bp
BA000032	Vibrio parahaemolyticus RIMD 2210633 DNA, chromosome 2, complete sequence.	17 bp
AM946015	Streptococcus uberis 0140J complete genome.	17 bp

Searched on August 8, 2010 (BLASTN 2.2.24)

Gene with base sequence highly analogous to the inserted 26-bp oligonucleotide (Table 2)

Genes with base sequence highly homologous to the inserted 26-bp oligonucleotide were SlpX for *Lactobacillus amylovorus* (homologous for 19 bp out of 1536 bp: S-Layer protein), peptidase S16 lon domain protein for *Thermodesulfobium narugense* (homologous for 17 bp out of 2382 bp: peptidase), Excinuclease ABC subunit C for *Shewanella* sp (homologous for 17 bp out of 1830 bp: nuclease), imisazole glycerol phosphate synthase for *Clostridiales* sp. (homologous for 17 bp out of 606 bp: transferase), and hypothetical protein for *Vibrio* sp. and *Vibrio parahaemolyticus* (homologous for 17 bp out of 153 bp: protein with unknown function). For *Staphylococcus aureus* (homologous for 18 bp out of 26,016 bp) and *Streptococcus uberis* (homologous for 17 bp out of 1,852,352 bp), it was confirmed not to be specifically functioning as a gene.

Table 2. Gene with base sequence highly analogous to the inserted 26-bp oligonucleotide

GenBank registration name	Name of gene product	Gene function	No. of homologous base / total number of base
Lactobacillus amylovorus GRL 1118, complete genome.	SlpX	S-Layer protein	19bp/1536bp
Staphylococcus aureus plasmid SAP063A, complete sequence.	Non-coding region	none	18bp/26,016bp
Thermodesulfobium narugense DSM 14796, complete genome.	Peptidase S16 lon domain protein	Peptidase	17bp/2382bp
Shewanella sp. MR-4, complete genome	Excinuclease ABC subunit C	Nuclease	17bp/1830bp
Clostridiales sp. SSC/2 draft genome.	imisazole glycerol phosphate synthase	Transferase	17bp/606bp
Vibrio sp. Ex25 chromosome 2, complete sequence.	hypothetical protein	Protein with unknown function	17bp/153bp
Vibrio parahaemolyticus RIMD 2210633 DNA, chromosome 2, complete sequence.	hypothetical protein	Protein with unknown function	17bp/153bp
Streptococcus uberis 0140J complete genome.	Non-coding region	none	17bp/1,852,352bp

Searched on April 11, 2012 (BLAST2.2.26)

Summary and discussions:

It was confirmed that the 26-bp oligonucleotide inserted into the *aroA* gene deleted avian *E.coli* strain EC34195 was not included in the gene of any *E.coli* or other bacteria registered to GenBank as of now.

Additionally, while genes with base sequence highly homologous to the inserted 26-bp oligonucleotide were found, the homologous 17-bp to 19-bp base sequences were merely base sequences of specific genes or a part of non-coding region, and it is unlikely the case that the inserted 26-bp base sequence functions as a gene of pathogenesis factor etc.

Attachment 8. Safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 in chicken (spray administration)

Objective:

The safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 were investigated by spray administering to newborn SPF chicks at 2.217×10^8 CFU/dose using *aroA* gene deleted attenuated avian *E.coli* live vaccine where the relevant bacterial strain is the main agent.

Test method:

A live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent was administered to 50 SPF chicks aged 1 day by coarse-spraying at a dose of 2.217×10^8 CFU/chick. No vaccine was administered to 25 SPF chicks as the cohabitating control group (Table 1). All chicks were visually investigated for any clinical signs on a daily basis.

Autopsy was conducted for 10 chicks from the vaccine administered group and 5 chicks from the cohabitating control group 4, 8, 11, 15, and 22 days after vaccination. At the time of autopsy, to recover bacteria from each chick, swab samples were collected from heart, liver and air sac, and cultured on MacConkey agar media.

Bacteria collected from each chick were identified using API 20E strips (identification kit) to identify *E.coli*. Additionally, investigation was made on no growth of the bacteria on basal media not containing aromatic amino acids (phenylalanine, tyrosine and tryptophan) and *p*-aminobenzoate (PABA). As such, investigation was made on the presence of the *aroA* gene deleted avian *E.coli* strain EC34195.

Swab samples of environmental samples were also collected from the isolator used for breeding, and presence of vaccine strain was monitored each time.

Table 1. Test design

Test group	Vaccine	Dose (dose per chick)	Route of vaccination*	Number of chicks**
1	*aroA* gene deleted attenuated avian *E.coli* live vaccine	2.217×10^8 CFU	Coarse spray	50
2	none	none	none	25

* A box for spraying was prepared for spray administration. Fifty chicks were gathered in the box, sprayed, and then placed in an isolator.

** Two isolators (#32 and #17) were prepared to accommodate 50 chicks from the vaccinated group and 25 chicks from the cohabitating control group. Each isolator accommodated 25 chicks from the vaccinated group and 12 or 13 chicks from the cohabitating control group.

Results:

No clinical signs of disease were observed for both vaccinated chicks and cohabitating control chicks. Additionally, no case of mortality attributable to the vaccination was observed during the period of study.

The vaccine strain was recovered from environmental samples collected 4 and 8 days after vaccination, yet the strain was not recovered from samples collected 11, 15, and 22 days after vaccination (Table 2).

At the time of autopsy 4 days after vaccination, the vaccine strain was recovered from 1 chick in the vaccinated group. At the time of autopsy 8, 11, 15 and 22 days after vaccination, the vaccine strain was not recovered from any chick of the vaccinated group. The vaccination strain was not recovered from chick of cohabitating control group at any time of the study (Table 3).

Bacteria such as wild *E.coli*, *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia liquefaciens* and *Fluorescent Pseudomonas* were recovered from the vaccinated group, control group, and the environmental samples at various time of the study.

Table 2. Results of bacterial isolation inside isolators

Time of isolation (day)	Isolator #	Isolation location	MacConkey media	Agar media	API identification kit	Trypticase soy agar	Identification
4	32	Top/front surface	−	nt*	nt	nt	NA**
		Waterer	+	+	*E.coli*	+	Wild-type
		Feeder	+	−	*E.coli*	+	Vaccine
		Floor surface	+	+	*E.coli*	+	Wild-type
	17	Top/front surface	−	nt*	nt	nt	NA**
		Waterer	+	+	*E.coli*	+	Wild-type
		Feeder	+	−	*E.coli*	+	Vaccine
		Feeder	+	−	*E.coli*	+	Vaccine
		Floor surface	+	+	*E.coli*	+	Wild-type
8	32	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	*Ent. cloacae, C.freundi*	+	*Ent. cloacae, C.freundi*
		Waterer	+	+	*E.coli*	+	Wild-type
		Feeder	+	+	*E.coli*	+	Wild-type
		Floor surface	+	+	*E.coli*	+	Wild-type
		Floor surface	+	+	*Ent. cloacae, Cit.freundi*	+	*Ent. cloacae, Cit.freundi*
	17	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	*Ent. cloacae, Cit.freundi*	+	*Ent. cloacae, Cit.freundi*
		Waterer	+	+	*E.coli*	+	Wild-type
		Feeder	−	nt	nt	nt	NA
		Floor surface	+	−	*E.coli*	+	Vaccine
		Floor surface	+	+	*E.coli*	+	Wild-type
11	32	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	No ID***	+	No ID
		Waterer	+	+	*Ent. c loacae,*	+	*Ent. c loacae,*
		Feeder	+	+	*Cit.freundi*	+	*Cit.freundi*
		Feeder	+	+	*E.coli*	+	Wild-type
		Floor surface	+	+	*E.coli*	+	Wild-type
	17	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	No ID	+	No ID
		Feeder	+	−	*E.coli*	+	Wild-type
		Floor surface	+	+	*E.coli*	+	Wild-type
15	32	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	*E.coli*	+	Wild-type
		Waterer	+	+	No ID	+	No ID
		Feeder	+	+	*E.coli*	+	Wild-type
		Floor surface	+	+	*E.coli*	+	Wild-type
	17	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	*Ent. cloacae, Cit.freundi Ser.liquifaciens*	+	*Ent. cloacae, Cit.freundi Ser.liquifaciens*
		Feeder	−	nt	nt	nt	NA
		Floor surface	+	+	No ID	+	No ID
22	32	Top/front surface	−	nt	nt	nt	NA
		Waterer	+	+	No ID	+	No ID
		Waterer	+	−	No ID	+	No ID
		Waterer	+	−	No ID	+	No ID
		Waterer	+	+	E.coli	+	Wild-type
		Waterer	+	+	*Ent. cloacae, Cit.freundi*	+	*Ent. cloacae, Cit.freundi*
		Feeder	−	nt	nt	nt	NA
		Floor surface	+	+	*E.coli*	+	Wild-type
	17	Top/front surface	−	nt	nt	nt	NA
		Waterer	−	nt	nt	nt	NA
		Feeder	−	nt	nt	nt	NA
		Floor surface	−	nt	nt	nt	NA

*: Not tested
**: Identification not carried out
***: Identification not possible

Table 3. Results of bacterial isolation from chicks by autopsy

Time of isolation (day)	Isolator #	No. of chicks isolated / No. of chicks tested		MacConkey media	Agar media	API identification kit	Trypticase soy agar	Identification
4	32	1/9		+	+	*E.coli*	+	Wild-type
	17	3/11	(1/11)*	+	+	*E.coli*	+	Vaccine
			(2/11)*	+	+	*E.coli*	+	Wild-type
8	32	3/11	(2/11)*	+	+	*E.coli*	+	Wild-type
			(1/11)	+	+	*Ent. cloacae, Cit.freundi*	+	*Ent. cloacae, Cit.freundi*
	17	0/7		−	nt**	nt	nt	NA***
11	32	0/7		−	nt	nt	nt	NA
	17	0/8		−	nt	nt	nt	NA
15	32	1/9		+	+	*Flu.pseudomonas*	+	*Flu.pseudomonas*
	17	1/7		+	+	*E.coli*	+	Wild-type
22	32	0/7		−	nt	nt	nt	NA
	17	0/8		−	nt	nt	nt	NA

*: Isolated from vaccinated chicks (other isolation cases are from cohabitating control chicks)
**: Not tested
***: Identification not carried out

Summary and discussions:

It was clarified that the live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent rapidly disappeared from chicks and the environment when coarse spray administered.

After coarse spray administration, the vaccine strain was recovered from only one chick in the vaccinated group 4 days after vaccination. The strain was recovered from environmental samples 4 and 8 days after vaccination, and not recovered in later days.

During the period of 3 weeks after vaccination, the vaccine strain was not collected from chicks of the cohabitating control chicks at any time.

From these, it was confirmed that this vaccine is safe to vaccinated chicks, cohabitating chicks and the environment when administered to SPF chicks aged 1 day by coarse-spaying.

Attachment 9. Safety of the *aroA* gene deleted avian *E.coli* strain EC34195 in mice

Objective:

The safety of the *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent was investigated using mice, animal outside the scope of application of the vaccine.

Test method:

Sixteen mice were used for testing with dividing into 2 groups. The *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent was given to 8 mice in Test Group 1 through intraperitoneal inoculation to at a dose of 5.0×10^6 CFU/mouse (0.5 mL) and to 8 mice in Test Group 2 through intracerebral inoculation at a dose of 5.0×10^6 CFU/mouse (0.03 mL), and the mice were observed on a daily basis for 7 days (Table 1).

Table 1. Test design

Test group	Vaccine	Dose (dose per mouse)	Route of vaccination	No. of mice
1	*aroA* gene deleted attenuated avian *E.coli* live vaccine	5.0×10^6 CFU (0.5 mL)	Intraperitoneal	8
2	*aroA* gene deleted attenuated avian *E.coli* live vaccine	5.0×10^6 CFU (0.03 mL)	Intracerebral	8

Results:

For the intraperitoneal administration group, no adverse events (death or other clinical sign) attributable to vaccination were observed during the period of observation. For the intracerebral administration group, while 1 mouse died 1 day after inoculation, it was considered to be due to an injury at the time of inoculation. For other 7 mice, no undesirable reaction (death or other clinical sign) attributable to vaccination was observed during the period of observation (Table 2).

Table 2. Safety evaluation of *aroA* gene deleted attenuated avian *E.coli* live vaccine in mice

Test Group	Route of administration	No. of animals	Mortality	Side reaction	Post-vaccination observation (days elapsed)						
					1	2	3	4	5	6	7
1	Intraperitoneal	8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
2	Intracerebral	8	1/8*	0/7	1/8	0/7	0/7	0/7	0/7	0/7	0/7

*: Death 1 day after inoculation (due to injury at the time of inoculation)

Summary and discussions:

The results above confirm the safety of the *aroA* gene deleted avian *E.coli* strain EC34195 when administered to mice, animal outside the scope of application of the vaccine, either by intraperitoneal inoculation or intracerebral inoculation.

Attachment 10. Safety of the *aroA* gene deleted avian *E.coli* strain EC34195 in pigs

Objective:

The safety of the *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent was investigated using pigs, animal outside the scope of application of the vaccine.

Test method:

The *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent was given to ten piglets aged 3 weeks through oral administration at a dose of 3.55×10^8 CFU/piglet (1.0 mL). Five non-vaccinated control piglets were bred in a separate house. All piglets were observed on a daily basis for clinical signs and death (Table 1).

Autopsy was carried out 3 weeks after vaccination (6-week old), organs (lungs, heart, liver and spleen) of piglets were assessed for any lesion arising from *E.coli*, and bacterial isolation was carried out.

Swab samples from each organ were cultured on MacConkey agar media for isolating *E.coli*, and all the plates that exhibited positive results were tested using API strips (identification kit) to confirm the presence of *E.coli*.

Table 1. Test design

Test group	Vaccine	Dose (dose per piglet)	Route of vaccination	No. of piglets
1	*aroA* gene deleted attenuated avian *E.coli* live vaccine	3.55×10^8 CFU (1.0 mL)	Oral	10
2	none	none	none	5

Results:

Table 2. Safety evaluation of *aroA* gene deleted attenuated avian *E.coli* live vaccine in piglets aged 3 weeks

Test Group	Route of administration	No. of animals	Mortality (%)	Lesion				Isolation of *aroA*– E.coli			
				Lung	Heart	Liver	Spleen	Lung	Heart	Liver	Spleen
1	Oral	10	10 (1/10*)	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
2	-	5	20 (1/5**)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

*: Death of vaccinated piglet due to renal lesion
**: Death of non-vaccinated piglet due to serious acute pneumonia

After the test, tissues (lungs, heart, liver and spleen) of piglets were collected for bacteria isolation. The vaccine strain was not recovered from piglets of the vaccinated group. Various kinds of unidentifiable bacteria were recovered from piglets of the non-vaccinated control group.

About the case of death

One piglet (#W567) in the vaccinated group and one piglet (#W551) in the non-vaccinated group were found dead on the 15th day and 20th day after vaccination, respectively. These 2 dead piglets were investigated for gross lesion by autopsy.

The death of the piglet (#W567) in the vaccinated group was caused not by vaccination but by kidney failure due to congenital renal disease.

The death of the piglet (#W551) in the non-vaccinated group was caused by serious acute pneumonia.

Post-mortem bacteria isolation

Tissues (lungs, heart, liver and spleen) of the 2 dead piglets (#W567 and #W551) were collected for bacteria isolation. Various kinds of unidentifiable bacteria were recovered from the piglet (#W551) in the non-vaccinated control group. No bacteria were recovered from the piglet (#W567) in the vaccinated group, including the *aroA* gene deleted avian *E.coli* vaccine strain.

Summary and discussions:

When administered to animals outside the scope of application of the vaccine (pigs), no adverse events (death or other clinical signs) attributable to the vaccine were observed. Additionally, autopsy did not find any gross lesion arising from *E.coli* in organs of all vaccinated piglets, and the *aroA* gene deleted avian *E.coli* was not recovered either (Table 2). Death was observed for a vaccinated piglet during the period of study, where the cause originated not from the vaccine but from kidney failure due to congenital renal disease.

The results above confirm the safety of the *aroA* gene deleted avian *E.coli* strain EC34195 when orally administered to pigs, animal outside the scope of application of the vaccine.

Attachment 11. Veterinary biologics risk assessment of Poulvac *E. coli* in the U.S.

This material is an extract of matters related to risk analysis from the final draft of "Veterinary Biologics Risk Assessment for the Field Testing and Licensure of *Escherichia Coli* Vaccine, Live Culture" by the Center for Veterinary Biologics, Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA) [Reference Material 11].

I. Summary (omitted)

II. Introduction

A. Veterinary biologics risk assessment process

When the USDA APHIS examines an application for transportation and release from containment of experimental veterinary biologics pursuant to the provisions of 9CFR 103.3, the Center for Veterinary Biologics Risk Analysis Process is used. Risk assessment performed in the process is designed for veterinary biologics, and employs multi-factorial approach to the assessment of risks on animal health, public health and the environment. The risk assessment includes rating of risks. Definition of risk is the standard definition, namely, "Risk refers to incidence rate of adverse events, and the consequence for when such adverse events occur". It requires announcement and documentation of a wide range of scientific analysis, mutual assessment and examination process. This approach is consistent with the standards for general risk assessment procedures.

In the risk assessment of veterinary biologics, focuses are placed on the characteristics of the vaccine strain and research environment. The safety of the vaccine strain bases on specific empirical data and evidenced scientific information.

B. Objective

The objective of the risk assessment is to identify the safety risk of environmental release of Category II, *Escherichia Coli* Vaccine, Live Culture.

The goal of this assessment is to guarantee the points below.

1) This test vaccine is safe to administer under outdoor conditions in controlled tests.
2) This vaccine is safe to license for general marketing and sales.

From the provisions of the Virus-Serum-Toxin Act (enacted in 1913, revised in 1985), USDA needs to guarantee that veterinary biologics are pure, safe and effective, as well as not worthless, contaminated, risky, or hazardous.

C. Recommendations (omitted)

III. Characteristics of Gene-deleted Organism (omitted)

IV. Risk Analysis

A. Characteristics of adverse events

Identification of adverse events is conducted for all adverse events that potentially occur against the animal health, public health and environment safety in relation to the field testing of the *aroA− E.coli* vaccine submitted for application.

1. Animal health safety

Field safety testing of the *Escherichia Coli* Vaccine, Live Culture, VS Code 1551.R0 submitted for application does not raise concerns over animal health safety. This vaccine was produced from the master seed. It has been clarified that the *aroA− E.coli* strain EC34195 is safe when inoculated to chickens and pigs. Additionally, for this vaccine, a safety study in mice as described in 9CFR113.33 was conducted, by FDAH, and passed. Therefore, it is assumed that release of this vaccine strain submitted for application does not induce adverse events to affect the animal health safety.

2. Public health safety

a. Summary

The safety of the master seed to humans has not been assessed. However, it is assumed that field safety testing for the *Escherichia Coli* Vaccine, Live Culture, VS Code 1551.R0 submitted for application using this nutrient requiring strain does not cause adverse events to the public health.
In case of human exposure, the principle of attenuation is expected to effectively function on humans as well.

b. Likelihood of human exposure

Humans exposed to this vaccine are limited to those involved in vaccination of poultry. Additionally, spray is planned to be conducted inside a sealed cabinet, and exposure to the vaccine is expected to be minimal. Further, the facts that replication of the vaccine strain inside the body of administered chickens and in the environment was limited and that no vaccine strain was detected for cohabitating chickens which can come in contact with administered chickens indicate a low potential of the vaccine spreading in the environment. Therefore, it can be said that human exposure to the vaccine is minimal.

c. Pathogenicity of parent strain to humans

Wild *E.coli* as the parent strain was isolated from cases of sepsis in chicken. Pathogenicity of the parent strain to humans is unknown.

d. Pathogenicity of vaccine strain to humans

MEDLINE search themed "*aroA* deletion mutants" discovered that two studies have been reported including information on human exposure to *aroA* deletion mutants. Abstracts of the two reports registered in MEDLINE are shown below.

i) (Stocker BA. Auxotrophic *Salmonella typhi* as live vaccine. Vaccine 1988; 6:141-145.)
"*Salmonella typhi* 541Ty has deletions at *aroA* and *purA*, causing requirement for aromatic metabolites (including *p*-aminobenzoate) and for adenine. None of 36 volunteers who drank 10^8 to 10^{10} bacteria of 541Ty or its Vi-negative mutant 543Ty showed any adverse effect; all gave evidence of cellular immune response but only a few had serum titre increases. *S. typhimurium* experiments (at the Wellcome Research Laboratories and at Stanford University) show that adenine requirement may reduce both bacterial survival in mouse tissues and live-vaccine efficacy. *S. typhi* attenuated only by block(s) in aromatic biosynthesis may be more effective as oral-route live vaccine."

ii) (Levine MM, Herrington D, Murphy JR, Morris JG, Losonsky G, Tall B, Lindberg AA, Svenson S, Baqar S, Edwards MF, et al. Safety, infectivity, immunogenicity, and in vivo stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. J Clin Invest 1987, 79:888-902.)

"Two *Salmonella typhi* mutants, 541Ty (Vi+) and 543Ty (Vi-), auxotrophic for *p*-aminobenzoate and adenine, were evaluated as live oral vaccines. 33 volunteers ingested single doses of 10^8, 10^9, or 10^{10} vaccine organisms, while four others received two 2 x 10^9 organism doses 4 d apart. No adverse reactions were observed. Vaccine was recovered from coprocultures of 29 of 37 vaccinees (78%) and from duodenal string cultures of two; repeated blood cultures were negative. The humoral antibody response to *S. typhi* O, H, Vi, and lysate antigens in serum and intestinal fluid was meager. In contrast, all vaccinees manifested cell-mediated immune responses. After vaccination, 69% of vaccinees overall and 89% of recipients of doses greater than or equal to 10^9 responded to *S. typhi* particulate or purified O polysaccharide antigens in lymphocyte replication studies but not to antigens of other Salmonella or *Escherichia coli*. All individuals, postvaccination, demonstrated a significant plasma-dependent mononuclear cell inhibition of wild *S. typhi*."

However, the master seed has not been assessed for humans. Therefore, the pathology of the strain to human is unknown. While the mutation in *aroA* induces requirements for aromatic metabolites, aromatic metabolites do not exist in tissues of vertebrates including humans and its replicability inside mammals is certainly low.

e. Potential effects of human exposure

The master seed has never been inoculated to humans. The actual effects of accidental human exposure are unknown. However, considering the biological traits of *aroA− E.coli* strain EC34195 where *aroA* is mutated, human exposure is not expected to raise safety concerns. Due to the mutation in *aroA*, its replicability inside mammal tissues has certainly be reduced.

3. Environmental safety

The vaccine used for field safety testing of *Escherichia Coli* Vaccine, Live Culture, VS Code 1551.R0 is planned to be produced from a master seed. The master seed was produced using wild *E.coli* similar to *E.coli* that exist in any poultry farm. The vaccine strain is not a strain where new genetic material was introduced to wild parent strain. Therefore, introduction of this vaccine to the environment does not cause introduction of new genetic codes to the environment. In fact, the vaccine strain lacks in a functionally important metabolic pathway, and introduction to organisms other than directly vaccinated chickens is at an ignorable level. It is demonstrated that, similar to *aroA−* Salmonella strain, replication of the vaccine strain does not continue unless nutrients are artificially supplied, and there hardly is a possibility to cause changes to the phenotype or gene. Experiments using various experimental animals in laboratories have demonstrated that the replicability of the vaccine strain in the nature is extremely low, and the phenotype is stable even when passaging is conducted over the maximum number of passage generally approved for vaccine production under ideal conditions and with successive supply of nutrients.

Field testing of this vaccine submitted for application does not raise concerns over the environmental safety. Even an unlikely event of accidental release of the vaccine strain is expected not to cause adverse events to the environment.

B. Assessment of release

1. Location of test hatchery

The identification information and location of hatcheries planned for conducting the test have been determined. The field safety test will be conducted only at one hatchery per participant. The identification information of hatcheries can only be confirmed by each participant at the time of determining the schedule of the field safety test.

2. Location of test poultry farm

The identification information and location of poultry farms planned for conducting the test have been determined. The field safety test will be conducted only at one poultry farm per participant. The identification information of poultry farms can only be confirmed by each participant at the time of determining the schedule of the field safety test.

3. Characteristics of test facilities

Test facilities are to be large-scale commercial hatcheries and poultry farms managed by typical breeding methods.

4. Persons responsible of the testing

Test requestor

Fort Dodge Animal Health

800 5th Street N.W.

Fort Dodge, Iowa 50501

Test monitor

[Private information and nondisclosed]

5. Test design

a. Number of testing animals

No less than 2,000 birds per hatchery (or poultry house)

b. Disposition of testing animals

Method employed at poultry farms

c. Route of administration

Spray administration during the period of newborn

d. Dose

Vaccine is dissolved to an appropriate volume in accordance with instructions on spray administration

e. Total dose of test vaccine

No less than 2,000 doses per hatchery (or poultry house)

f. Frequency and period of exposure

One portion is to be administered during the period of newborn.

g. Waste disposal method

Waste disposal method at test facilities is to comply with the usual practice at each poultry farm.

h. Decontamination of test facilities

Decontamination method at test facilities is to comply with the usual practice at each poultry farm.

6. Possibility of release to and spread in the environment

From its molecular and biological traits, the survivability of the vaccine strain under conditions lacking aromatic metabolites is limited and its possibility to be released, spread and establish in the environment is low. Even if the vaccine strain spreads via media during transportation of chickens, it is totally unlikely the case that it is released to the environment and to establish.

7. Possibility of establishing in the environment

From its molecular and biological traits, there is no possibility for the vaccine strain to establish in the environment. In addition, *aroA* mutation is considered to be certainly reducing its replicability in the environment.

8. Monitoring

Observation is planned to be conducted in accordance with the field test plan approved by USDA.

9. Risk management plan for adverse events

Any adverse events that occur during the period of testing are to be immediately notified to the responsible persons and to USDA. When deemed having serious risk to other animals, personnel or the environment, the area will be designated as “off limit”. The antibiotic sensitivity of *aroA− E.coli* strain EC34195 has been confirmed. Therefore, swift and effective use of antibiotic treatment can reduce adverse effects in case of undesirable human exposure.

C. Examination of risks

1. Examination of risks associated with the possibility of reversion to virulence

a. Likelihood Rating

The likelihood of risk on animal health safety, public health safety and environmental safety is rated based on the following standards:

Low = Adverse events are unlikely to occur;

Medium = Adverse events may occur; or,

High = Adverse events are highly likely to occur.

The likelihood of the *aroA− E.coli* strain EC34195 reverting to virulence is rated "Low = Adverse events are unlikely to occur".

The basis of the rating has been claimed in this assessment many times. The deletion of *aroA* gene was described in a. of III.A.2 where the characteristics of genetic deletion were discussed. In the *aroA− E.coli* strain EC34195, the *aroA* gene that codes 5-enolpyruvyl shikimate-3-phosphate synthetase is site-specifically deleted. This means that the *aroA− E.coli* strain EC34195 needs to obtain gene from external sources through recombination. The chance for the *aroA− E.coli* strain EC34195 to convert to a strongly virulent strain is practically zero.

b. Consequence Rating

Consequence on animal health safety, public health safety, and environmental safety is also rated based on the following standards:

Low = Impact of adverse event is small
(Adverse events naturally disappear and the impact is minor)

Medium = Impact of adverse event is moderate
(Adverse event have an impact, but the impact is not permanent and can be dealt)

High = Impact of adverse event is large
(Adverse events have an impact, and the impact is permanent and cannot be dealt)

The consequence in case of the *aroA− E.coli* strain EC34195 reverting to virulence is rated "Low = Impact of adverse event is small (Adverse events naturally disappear and the impact is minor)".

Currently, the most effective control method of colibacillosis is preventive use of antibiotics. However, as a result, wild *E.coli* groups have acquired high-level resistance to antibiotics, and the occupancy of resistant strains in the environment is rising. Even if the *aroA− E.coli* strain EC34195 reverts to virulence, it is unlikely the case that its pathogenicity is higher than the pathogenicity of endogenous *E.coli* residing in the area. When animals are infected with the vaccine strain reverted to virulence, it is unlikely the case that the symptoms become worse than when infected with wild strains. The antibiotic sensitivity profile of the vaccine strain has practically been confirmed, and it is possible to swiftly provide infected animals with veterinary treatment.

c. Certainty Rating

Likelihood Rating and Consequence Rating are adjusted by Certainty Rating based on the following standards:

Certain = Rating is directly backed by scientific basis;

Moderately certain = Rating is indirectly backed by scientific basis; and,

Uncertain = Rating is not backed by scientific basis.

The certainty of the possibility of the *aroA− E.coli* strain EC34195 reverting to virulence is rated "Certain = Rating is directly backed by scientific basis".

The *aroA− E.coli* strain EC34195 has been evaluated for reversion to virulence, discharge and propagation capacity. From the fact that the *aroA− E.coli* strain EC34195 was not recovered from cohabitating control groups for any passage, it is considered that the *aroA− E.coli* strain EC34195 did not propagate to adjoining chickens. The number of live *aroA− E.coli* strain EC34195 in the pooled suspension did not exhibit an increase for three passages either.

In addition to the above, no death attributable to the *aroA− E.coli* strain EC34195 occurred during the period of the study, reaching a conclusion that three-generation *in vivo* passaging of the *aroA− E.coli* strain EC34195 on chickens did not cause reversion to virulence. (A report titled "Reversion to Virulence Study of Modified Live *Escherichia coli* Vaccine for Use in Chickens" submitted to CVB on October 28, 2002)

d. Calculation of expected risks associated with the possibility of reversion to virulence

The expected risks associated with the possibility of reversion to virulence can be calculated from the information above. The expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994) is calculated using the following information:

Likelihood "Low" LL = 1.00

Consequence "Low" CL = 1.00

Degree of Certainty Rating II (Degree of Certainty Rating I is used only when the likelihood is rated medium or high and the consequence is rated medium or high).

Certainty C = 1.00

Expected risk = [(Likelihood) x (Certainty)] x [(Consequence) x (Certainty)]

(1.00 x 1.00) x (1.00 x 1.00) = 1.00

Therefore, the expected risk is calculated to be 1.00.

e. Risk Rating

Risk Rating is determined referencing the expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994). Since the expected risk is 1.00, the Risk Rating is:

Low = Allowable risk (Note: Consequence is minor and likelihood is extremely low)
Level of concern caused by the application is extremely low
(no reason to reject the application)

2. Examination of risks associated with the possibility of discharge to and spread in the environment

a. Likelihood Rating

The likelihood of risk on animal health safety, public health safety and environmental safety is rated based on the following standards:

Low =	Adverse events are unlikely to occur;
Medium =	Adverse events may occur; or,
High =	Adverse events are highly likely to occur.

The likelihood of the *aroA*− *E.coli* strain EC34195 being discharged to and spreading in the environment is rated "Low = Adverse events are unlikely to occur".

In Item 2 where explanations are given on the master seed, it was exhibited that the vaccine strain did not propagate from vaccinated chickens to unvaccinated adjoining chickens in the study on discharge and propagation conducted by FDAH. FDAH is currently developing live *E.coli* vaccine using the *aroA*− *E.coli* strain EC34195. Even if the vaccine strain is discharged from vaccinated chicken, the mutation in *aroA* has made it requiring aromatic compounds [tyrosine, phenylalanine, tryptophan, *p*-aminobenzoate (PABA) and 2,3-dihydroxybenzoate (DHBA)] and unable to multiply unless the aromatic compounds are supplied, and it is unlikely the case that the strain will remain in the environment.

Because the vaccine strain has mutation in the chromosomal DNA, it is unable to multiply in the environment unless freely available essential aromatic compounds are supplied. Therefore, even if the vaccine strain is released to the environment, the strain is considered not to survive and spread throughout the environment.

b. Consequence Rating

Consequence on animal health safety, public health safety, and environmental safety is also rated based on the following standards:

Low =	Impact of adverse event is small (Adverse events naturally disappear and the impact is minor)
Medium =	Impact of adverse event is moderate (Adverse events have an impact, but the impact is not permanent and can be dealt)
High =	Impact of adverse event is large (Adverse events have as impact, and the impact is permanent and cannot be dealt)

The consequence of discharge of the *aroA*− *E.coli* strain EC34195 to the environment and spreading is rated "Low = Impact of adverse event is small (Adverse events naturally disappear and the impact is minor)".

The basis of the Consequence Rating "Low" is shown in the item above on the likelihood. Even if the *aroA*− *E.coli* strain EC34195 is released to and spread in the environment, it is considered that the event will automatically disappear and the impact is minor.

c. Certainty Rating

The certainty in regard to animal health safety, public health safety and environmental safety is also rated based on the following standards:

Certain = Rating is directly backed by scientific basis;

Moderately certain = Rating is indirectly backed by scientific basis; and,

Uncertain = Rating is not backed by scientific basis.

The certainty of the unlikeliness of the *aroA− E.coli* strain EC34195 being released to and spreading in the environment is rated “Certain = Rating is directly backed by scientific basis”.

There is a possibility of the *aroA− E.coli* strain EC34195 to be released into the environment due to accidental overdose, fracture of vaccine vials, or similar events. However, for its enzyme pathway being mutated, it is unlikely the case that the strain spreads in the environment. Its spread in the environment is considered to require reversion to virulence. The scientific basis of the unlikeliness of such an event taking place is discussed in III.B.2.d. Since the deletion in the *aroA* gene is site specific, the probability of the *aroA− E.coli* strain EC34195 reverting to the parent strain is practically zero.

d. Calculation of expected risks associated with the possibility of release to and spread in the environment

The expected risks associated with the possibility of release to and spread in the environment can be calculated from the information above. The expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994) is calculated using the following information:

Likelihood “Low” LL = 1.00

Consequence “Low” CL = 1.00

Degree of Certainty Rating II (Degree of Certainty Rating I is used only when the likelihood is rated medium or high and the consequence is rated medium or high).

Certainty C = 1.00

Expected risk = [(Likelihood) x (Certainty)] x [(Consequence) x (Certainty)]

(1.00 x 1.00) x (1.00 x 1.00) = 1.00

Therefore, the expected risk is calculated to be 1.00.

e. Risk Rating

Risk Rating is determined referencing the expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994). Since the expected risk is 1.00, the Risk Rating is:

Low = Allowable risk

Level of concern caused by the application is extremely low

(no reason to reject the application)

3. Examination of risks associated with the possibility of establishing in the environment

a. Likelihood Rating

The likelihood of risk on animal health safety, public health safety and environmental safety is rated based on the following standards:

Low = Adverse events are unlikely to occur;

Medium = Adverse events may occur; or,

High = Adverse events are highly likely to occur.

The likelihood of the *aroA−* *E.coli* strain EC34195 establishing in the environment is rated "Low = Adverse events are unlikely to occur".

Even if the vaccine strain is discharged from vaccinated chickens, it requires aromatic compounds (tyrosine, phenylalanine, tryptophan, PABA and DHBA) due to the *aroA* mutation and is unable to multiply unless the aromatic compounds are supplied, and it is unlikely the case that the strain establishes in the environment.

The only method for the *aroA−* *E.coli* strain EC34195 to establish in the environment is reversion to virulence, and the likelihood of this event taking place is extremely low. The probability of the *aroA−* *E.coli* strain EC34195 reverting to the parent strain is practically zero.

b. Consequence Rating

Consequence on animal health safety, public health safety, and environmental safety is also rated based on the following standards:

Low = Impact of adverse event is small
(Adverse events naturally disappear and the impact is minor)

Medium = Impact of adverse event is moderate
(Adverse events have an impact, but the impact is not permanent and can be dealt)

High = Impact of adverse event is large
(Adverse events have an impact, and the impact is permanent and cannot be dealt)

The consequence of the *aroA−* *E.coli* strain EC34195 establishing in the environment is rated "Low = Impact of adverse event is small (Adverse events naturally disappear and the impact is minor)".

Scientific bases have proven the establishment of *E.coli* in the environment. The differences between *E.coli* already established in the environment and the *aroA−* *E.coli* strain EC34195 are the facts that the antibiotic sensitivity profile of the *aroA−* *E.coli* strain EC34195 have been confirmed, and there is no likelihood of the *aroA−* *E.coli* strain EC34195 establishing in the environment and the impact is considered minor.

c. Certainty Rating

The certainty in regard to animal health safety, public health safety and environmental safety is also rated based on the following standards:

Certain = Rating is directly backed by scientific basis;

Moderately certain = Rating is indirectly backed by scientific basis; and,

Uncertain = Rating is not backed by scientific basis.

The certainty of the unlikeliness of the *aroA−* *E.coli* strain EC34195 establishing in the environment is rated "Certain = Rating is directly backed by scientific basis".

The only method for the *aroA−* *E.coli* strain EC34195 to establish in the environment is reversion to virulence. The scientific basis of the unlikeliness of this event taking place is discussed in III.B.2.g. Since the deletion of the *aroA* gene is site specific, the probability of the *aroA−* *E.coli* strain EC34195 reverting to the parent strain is practically zero.

d. Calculation of expected risks associated with the possibility of establishing in the environment

The expected risks associated with the possibility of establishment in the environment can be calculated from the information above. The expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994) is calculated using the following information:

Likelihood “Low” LL = 1.00

Consequence “Low” CL = 1.00

Degree of Certainty Rating II (Degree of Certainty Rating I is used only when the likelihood is rated medium or high and the consequence is rated medium or high).

Certainty C = 1.00

Expected risk = [(Likelihood) x (Certainty)] x [(Consequence) x (Certainty)]

(1.00 x 1.00) x (1.00 x 1.00) = 1.00

Therefore, the expected risk is calculated to be 1.00.

e. Risk Rating

Risk Rating is determined referencing the expected risk in veterinary biologics risk assessment (Gay, C.G. & Orr, R.L., 1994). Since the expected risk is 1.00, the Risk Rating is:

Low = Allowable risk
Level of concern caused by the application is extremely low

V. Recommendations on risk management

A. Procedure

For risk management, means to reduce or eliminate animal health risk, public health risk and environmental risk shall be determined using the information shown in the items above.

B. Recommendations

Animal health risk, public health risk or environmental risk is low. Risk Rating “Low” indicates that the risk is acceptable and concerns arising from field testing by FDAH for the live *E.coli* vaccine submitted for application and subsequence licensing are minor. FDAH requests approval of field testing in accordance with the requirements of 9 CFR 103.3 and the approved test plan.

Attachment 12. Approval status and sales of Poulvac *E. coli* outside Japan

Approval status:

This vaccine is approved as a drug for animals in the U.S., Thailand, Malaysia and Philippines. The date of approval and sales amount are as shown in the Table below.

Country	Date of approval	Sales amount (dose)*
U.S.	April 2006	219,170,000
Thailand	February 2008	38,000,000
Malaysia	August 2009	33,000,000
Philippines	March 2010	9,884,900

* Sales amount after approval (as of April 2012)

Procedure of approval examination at Cartagena Protocol non-ratified country (U.S.) and ratified countries (Thailand, Malaysia, Philippines):

The development of Poulvac E.coli (brand name of Poulvac *E.coli* outside Japan) started with the Fort Dodge Animal Health (currently Zoetis) making an application to the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA). The procedure of approval examination was proceeded with in accordance with the Biotechnology-derived Veterinary Biologics Category II in guidelines stipulated by the Veterinary Service Memorandum (VSM) No. 800.205 (http://www.aphis.usda.gov/vs/cvb) in addition to the general procedures for application for New Biotechnology for Preparation of Animal Biological Products, and an approval was given in April 2006.

While the U.S. has not ratified the Cartagena Protocol, all preparations that fall under the category of genetically modified organisms (GMO) are obligated to undergo official Veterinary Risk Assessment by USDA. The Risk Assessment is conducted in a form of mutual evaluation by the applicant and the USDA APHIS. For Biotechnology-derived Veterinary Biologics, prior to conducting field trial, its molecular or biological characteristics are clarified, and the risk level (safety concerns) to animal health, public health and the environment is shown in the Summary Information Format (SIF) that includes Risk Assessment. For Poulvac E.coli, through Risk Assessment including field trial, its risk level to animal health, public health and the environment was assessed low (safety concerns are minor).

In Thailand, Malaysia and Philippines, countries that ratified the Cartagena Protocol, the SIF including Risk Assessment above was submitted on applying for approval. It is reported that there was no request or comment related to living modified organism (LMO).

However, in Philippines, field trail in the country is required for approval, and field trial was conducted at 5 commercial broiler farms under the supervision and guidance of the relevant authority. In Thailand and Malaysia, field trial in the country is not required for approval and was not requested.

Attachment 13. Reports on side effects of Poulvac *E.coli* outside Japan (by PV-Works)

As a result of search using PV-Works[1] (global pharmacovigilance search software) adopted by Pfizer Inc., no adverse events to inoculated chicken, human and other animals were found for Poulvac E.coli (brand name of Poulvac *E. coli* outside Japan) supplied in countries from January 2007 to date (April 25, 2012).

[1] Information on adverse events of animal health products of Pfizer Inc. gathered worldwide are stored in this database. It is possible to search information on adverse events by the product name, country name, etc. using this database.

Attachment 14. Safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 in chicken (ophthalmological administration) (overseas study)

Objective:

The safety, clearance, discharge and spread of the *aroA* gene deleted avian *E.coli* strain EC34195 were investigated by ophthalmologically administering to chicken.

Test method:

The *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent (Poulvac *E. coli*) was given to 50 chicks aged 1 day through ophthalmological administration at a dose of no less than 1.0×10^9 CFU/chick, and the vaccinated chicks were cohabitated with 25 non-vaccinated chicks.

The chicks were observed for clinical signs, and autopsy was periodically conducted to observe for gross lesion. Swab specimen from cloaca, nasal cavity and internal organs as well as environmental samples (bedding, feed, and drinking water) were chronologically collected for up to 21 days after vaccination, and isolation culturing of vaccine strain was conducted.

Results:

Clinical signs and autopsy findings

During the period of observation, no clinical sign or gross lesion attributable to the vaccination was observed.

Results of isolation of vaccine strain from internal organs

The vaccine strain was isolated from internal organs of one vaccinated chick 4 days after vaccination. The vaccine strain was not isolated from cohabitating chicks.

Isolation of vaccine strain from cloaca swab

The vaccine strain was isolated from cloaca swab for up to 14 days after vaccination for vaccinated chicks and for up to 11 days after vaccination for cohabitating chicks.

Isolation of vaccine strain from nasal cavity

The vaccine strain was isolated from nasal cavity only once for both vaccinated chicks and cohabitating chicks.

Isolation of vaccine strain from environmental samples

The vaccine strain was isolated from bedding and feed for up to 21 days after vaccination, and from drinking water for up to 7 days from vaccination.

Test data are confidential and nondisclosed.

Attachment 15. Discharge and lifetime of the *aroA* gene deleted avian *E.coli* strain EC34195 in the environment (overseas study)

Objective:

Discharge and lifetime of the *aroA* gene deleted avian *E.coli* strain EC34195 in the environment was investigated through administration to chickens by instilling in the eye or spraying.

Test method:

The *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent (Poulvac *E. coli*) was given to 20 chicks aged 1 day through ophthalmological or spray administration.
Chicks were observed on a daily basis for clinical signs, and autopsy was conducted for test chicks 42 days after vaccination to observe for gross lesion. Cloaca swab and environmental samples (bedding, feed, and drinking water) were collected for up to 42 days after vaccination, and isolation culturing of vaccine strain was conducted.

Results:

Clinical signs and autopsy findings

During the period of observation, no clinical sign or gross lesion was observed.

Isolation of vaccine strain from cloaca swab

Regardless of the route of administration (spraying or instilling), the vaccine strain was isolated from cloaca swab for vaccinated chicks for up to 28 days after vaccination.

Isolation of vaccine strain from environmental samples

When instilled, the vaccine strain was isolated from feed and drinking water for up to 28 days after vaccination. When sprayed, vaccine strain was isolated from bedding for up to 35 days after vaccination.

Test data are confidential and nondisclosed.

Attachment 16. Multicenter trial of Poulvac *E. coli* in Morocco

Source: Mombarg, M. et al.: Safety and efficacy of an *aroA*-deleted live vaccine against avian colibacillosis in a multicentre field trial in broilers in Morocco. Avian Pathology, 43, 276–281. (2014)

Objective:

The safety and efficacy of the *aroA* gene deleted attenuated avian *E.coli* live vaccine where the *aroA* gene deleted avian *E.coli* strain EC34195 is the main agent (Poulvac *E. coli*) for when administered to broilers outdoors were investigated by a multicenter field trial.

Test method:

The trial was conducted at 18 facilities (15 farms) in Morocco. The testing animal used was broilers, and administration of the trial drug was conducted at the age of 1 day at the farm or hatchery. To the vaccine group consisting of 112,490 chicks in total, Poulvac *E. coli* (1.3×10^8 CFU/chick) was spray administered at a dose of 200-220 mL per 1,000 chicks. To the control group consisting of 112,476 chicks in total, mineral water was given when paired to chicks vaccinated at a farm, similar to the vaccine, for the purpose of realizing blank testing. Administration of mineral water for the purpose of realizing blank testing was not conducted to control chicks when paired to chicks vaccinated at a hatchery, because chicks in the vaccinated group will be adequately dried by the time they arrive at the farm and become impossible to distinguish from chicks of the control group. The safety and efficacy were evaluated by observing chicks from the date of trial drug administration to the date of slaughter (aged 37 days to 53 days).

Table 1. Test design

Test group	Trial drug	Route of administration	Dose	No. of chicks
Vaccine group	Poulvac *E. coli*	Spray	200-220 mL/1000 chicks (1.3×10^8 CFU/chick)[2)]	112,490
Control group	Mineral water or no administration[1)]	Spray	200-220 mL/1000 chicks	112,476

1) Mineral water was given to the control group when paired to chicks vaccinated at a farm, similar to the vaccine group, for the purpose of realizing blank testing. Administration was not conducted to the control group when paired to chicks vaccinated at a hatchery, because chicks in the vaccinated group will be adequately dried by the time they arrive at the farm and become impossible to distinguish from chicks of the control group.
2) Poulvac *E. coli* was used by diluting with mineral water to be 1.3×10^8 CFU per chick (approx. 0.2 mL).

Among the safety variables, the average daily weight gain (ADWG), mortality, feed conversion rate (FCR) and the mean number of antibiotic treatment days were evaluated by comparing with the non-inferiority limit value (delta: set to 5% or –5%) for the upper or lower limit of 95% confidence interval (CI) of the difference between the vaccine group and the control group. No statistical analysis was conducted for clinical observations and post-mortem investigations.
Among the efficacy variables, the mortality, ADWG, FCR, the mean number of antibiotic treatment days, the percentage of birds marketed and the prevalence of colibacillosis-like lesions at slaughter were evaluated by comparing between groups using a general linear mixed model (alpha level of 0.05). No statistical analysis was conducted for clinical observations and post-mortem investigations.

Results:

Safety variables

Clinical observations of post-mortem investigation

No adverse events potentially attributable to administration of Poulvac *E. coli* were observed.

Mortality

Least-squares mean mortality for 14 days from the date of trial drug administration was 1.48% for both the vaccine group and the control group. The 95% CI was 1.14 to 1.88 for the vaccine group and 1.13 to 1.88 for the control group. The upper 95% CI of the difference between the vaccine group and the control group did not exceed the delta (set to 5%), confirming the non-inferiority of Poulvac *E.coli* administration for the mortality.

ADWG

Least-squares mean ADWG for 14 days from the date of trial drug administration was 19.1 g/day for the vaccine group and 18.9 g/day for the control group. The 95% CI was 17.4 to 20.9 for the vaccine group and 17.2 to 20.7 for the control group. The upper 95% CI of the difference between the vaccine group and the control group did not exceed the delta (set to –5%), confirming the non-inferiority of Poulvac *E. coli* administration for ADWG.

FCR

The least-squares mean FCR for 14 days from the date of trial drug administration was 2.14 for the vaccine group and 2.20 for the control group. The 95% CI was 1.78 to 2.50 for the vaccine group and 1.83 to 2.56 for the control group. The upper 95% CI of the difference between the vaccine group and the control group exceeded the delta (set to 5%), not confirming the non-inferiority of Poulvac *E. coli* administration for the FCR.

Efficacy variables

Mortality

Least-squared mean mortality from the date of trial drug administration to the date of slaughter was 9.3% for the vaccine group and 10.3% for the control group, exhibiting a significant difference between the two treatment groups ($P = 0.0203$) (Table 2).

ADWG

Least-squares mean ADWG from the date of trial drug administration to the date of slaughter was 47.8 g/day for the vaccine group and 46.2 g/day for the control group, exhibiting a significant difference between the two treatment groups ($P = 0.0006$) (Table 2).

FCR

The least-squares mean FCR from the date of trial drug administration to the date of slaughter was 2.18 for the vaccine group and 2.22 for the control group, not exhibiting a significant difference between the two treatment groups ($P = 0.1856$) (Table 3).

Table 2. Efficacy evaluation results (mortality and average daily weight gain)

Farm number[1)]	Number of chicks (at trial start)		Mortality (%)		ADWG (g/day)	
	V	C	V	C	V	C
1	5999	6002	9.6	11.0	48.9	48.1
3	8000	8000	4.6	6.6	52.5	51.7
5	6000	6000	2.3	2.1	58.4	54.0
6	6000	6000	16.4	18.2	38.5	37.1
7	6504	6502	5.1	7.2	48.5	46.7
8	6502	6494	9.9	11.4	48.1	47.0
9	5000	5000	5.9	6.9	51.5	47.8
10	5000	5000	20.7	20.6	40.8	39.8
11	5000	5000	22.4	22.2	42.1	39.6
12	4998	5003	5.9	5.9	43.5	42.6
13	8000	8000	28.4	28.9	45.8	45.1
14-1	5001	4991	6.0	10.1	46.8	45.1
14-2	7006	7004	9.0	10.8	45.5	43.8
15-1	6240	6240	5.6	4.2	n.a.	n.a.
15-2	6240	6240	3.2	4.3	n.a.	n.a.
16-1	8000	8000	9.3	8.1	51.1	51.0
16-2	8000	8000	5.8	5.9	50.6	52.0
17	5000	5000	5.2	7.6	53.9	51.6
Least-squares mean			9.3*	10.3	47.8*	46.2
(95% CI)			(5.9 to 13.5)	(6.7 to 14.6)	(44.8 to 50.9)	(43.2 to 49.3)

V: vaccine group. C: control group. n.a.: not available (excluded from efficacy evaluation for inadequate record)

1) Each farm contained paired poultry houses that were assigned either to vaccine or control treatment groups.

* Significantly different ($P \leq 0.05$) from control group.

Mean number of antibiotic treatment days

Since antibiotic treatment was conducted from 15 days after vaccination, evaluation was made for the treatment days from 15 days after vaccination to the date of slaughter. The least-squares mean number of treatment days during the said period was 0.5 days for the vaccine group and 2.0 days for the control group, exhibiting a significant difference between the two treatment groups ($P = 0.0008$) (Table 3).

Percentage of birds marketed

The least-squares mean percentage of birds marketed was 90.0% for the vaccine group and 89.0% for the control group, exhibiting a significant difference between the two treatment groups ($P = 0.0309$).

Prevalence of colibacillosis-like lesions

The least-squares mean colibacillosis-like lesion score at slaughter was 1.7% for the vaccine group and 3.5% for the control group, exhibiting a significant difference between the two treatment groups ($P = 0.0054$) (Table 3).

Table 3. Efficacy evaluation results
(feed conversion rate, number of antibiotic treatment days and prevalence of colibacillosis-like lesions)

	FCR (kg/kg)		Number of antibiotic treatment days		Prevalence of colibacillosis-like lesions (%)	
Farm number[1)]	V	C	V	C	V	C
1	2.03	2.07	2	3	9.5	6.2
3	1.95	1.96	0	3	1.2	3.8
5	1.45	1.46	0	0	0.7	5.1
6	3.04	3.08	1	3	2.9	3.0
7	1.74	1.75	0	2	0.9	2.0
8	1.91	1.86	1	1	0.8	2.9
9	2.25	2.42	1	1	5.2	2.2
10	3.16	3.36	0	2	0.4	2.0
11	3.18	3.13	0	4	0.7	2.3
12	2.05	2.15	0	0	0.6	3.8
13	2.50	2.33	0	0	1.8	3.6
14-1	1.70	1.91	0	1	1.4	4.8
14-2	1.74	2.03	1	1	0.6	4.2
15-1	n.a.	n.a.	0	4	n.a.	n.a.
15-2	n.a.	n.a.	0	4	n.a.	n.a.
16-1	1.96	1.97	0	0	1.8	3.3
16-2	1.80	1.71	4	4	1.6	3.7
17	1.70	1.74	0	3	2.1	5.9
Least-squares mean	2.18	2.22	0.5*	2.0	1.7*	3.5
(95% CI)	(1.85 to 2.51)	(1.89 to 2.55)	(–0.2 to 1.3)	(1.3 to 2.7)	(1.0 to 2.6)	(2.5 to 4.7)

V: vaccine group. C: control group. n.a.: not available (excluded from efficacy evaluation for inadequate record)

1) Each farm contained paired poultry houses that were assigned either to vaccine or control treatment groups.

* Significantly different ($P \leq 0.05$) from control group.

Clinical observations and post-mortem investigations

Colibacillosis was suspected clinically and/or during post-mortem investigations in all farms for at least one point in time, except for Farms 5, 10, 11, 13 and 17. *E.coli* was isolated in samples taken from nine farms, and serotyping after identification of *E.coli* was performed in 13 cases, originating from eight farms. Results for this serotyping are summarized in Table 4.

Other clinical problems reported were coccidiosis (Farms 10 and 11), Gumboro disease (Farms 6 and 17), severe heat stress with high mortality (Farms 10, 11 and 13; with room temperatures exceeding 40°C), and digestive problems of unknown aetiology (Farms 9 and 10).

Table 4. Serotypes of isolated *E.coli*

Farm	Age of birds at sampling (days)	Treatment group	Serotype
1	29	Control	O78 Non-typeable
3	26	Vaccine	O78 O135 Non-typeable
3	26	Control	Non-typeable
6	13	Vaccine	O1 O135
7	34	Vaccine	Non-typeable
7	34	Control	O78 Non-typeable
8	35	Control	O78 O118 Non-typeable
9	20	Vaccine	O78
9	20	Control	O78
14-2	35	Control	O1 O118
16-1	43	Vaccine	O78
16-1	43	Control	O78 Non-typeable
16-2	37	Vaccine	O78

Summary and discussions:

No adverse events attributable to the administration of Poulvac *E.coli* were observed, and the non-inferiority of Poulvac *E.coli* administration was confirmed for average daily weight gain and mortality. From these results, the safety of Poulvac *E.coli* administration to newborn broiler chicks was confirmed.

Non-inferiority was not confirmed for the feed conversion rate. This is due to major variations in the estimates of feed conversion rate for 2 weeks from vaccination because feeding is conducted only once in several days for young chicks.

Regarding the efficacy, a significant different due to administration of Poulvac *E. coli* was observed in the prevalence of colibacillosis-like lesions at slaughter, average daily weight gain, the number of antibiotic treatment days, the percentage of birds marketed and the mortality, confirming alleviation of avian colibacillosis by administration of Poulvac *E. coli*. The results above confirm the safety and efficacy of outdoor use of Poulvac *E. coli*.