CLINICAL TRIAL REFERENCE EC-2013-CB-006 FOR THE ASSESSMENT OF THE VACCINE PB-35

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

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SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

A. General information

1. Details of notification

(a) Member State of notification	SPAIN
(b) Notification number	B/ES/13/21
(c) Date of acknowledgement of notification	03/09/2013
(d) Title of the project	Assessment of the safety and efficacy of the live vaccine PB-35 against Porcine Pleuropneumonia caused by *Actinobacillus pleuropneumoniae*
(e) Proposed period of release	From October 2013 to October 2015

2. Notifier

Name of institution or company	LABORATORIOS HIPRA, S.A.

3. GMO characterisation

(a) Indicate whether the GMO is a:

(a) Indicate whether the GMO is a:	Viroid	☐
	RNA virus	☐
	DNA virus	☐
	Bacterium	☒
	Fungus	☐
	Animal	☐
	- mammals	☐
	- insect	☐
	- fish	☐
	- other animal	☐ specify phylum, class
Other, specify (kingdom, phylum and class)		
(b) Identity of the GMO (genus and species) *Actinobacillus pleuropneumoniae*, strain HP-1967		

(c) Genetic stability – according to Annex IIIa, II, A(10)

When considering those factors involved in the genetic stability of the bacterium *Actinobacillus pleuropneumoniae*, it should be taken into account that several virulence factors have been described: the bacterial capsule, lipopolysaccharides (LPS) and toxins. *Actinobacillus pleuropneumoniae* produces four types of RTX toxins: ApxI, ApxII, ApxIII and ApxIV. The RTX toxins are codified by operon composed of four consecutive genes: *gene A, gene B, gene C and gene D.* Genes C and A are involved in the production of active toxin, whereas genes B and D are involved in the secretion of the active toxin. All serotypes described up to date produce ApxIV; serotypes 7, 10 and 12 produce an additional toxin and serotypes 1-6, 8, 9 and 11 produce 2 additional toxins. All these characteristics demonstrate the enormous variability between the different serotypes of *Actinobacillus pleuropneumoniae.*

Due to the importance of the virulence factors involved in the pathogenic course of the Porcine Pleuropneumonia and in the development of protective immunity against the disease, several studies have been conducted, which consist of the obtainment of mutant strains in which specific genomic deletions intended to inactivate determined toxins have been conducted. Thus the role of these inactivated toxins in the pathogenic and immunogenic capacity of the bacterium could have been evaluated. All these studies demonstrated the enormous genetic variability that the different strains of *Actinobacillus pleuropneumoniae* can show, and how the introduction of genomic mutations produces mutant strains capable of multiplying and spreading in the target animal.

On the other hand, the strain HP-1967 of *Actinobacillus pleuropneumoniae* shows a characteristic and specific restriction pattern, which can be easily identified. This pattern constitutes a characteristic “fingerprint” of this strain which permits both its characterisation and the detection of possible modifications in its genomic structure. This data indicates that we’re facing one bacterium provided with a well characterised and identifiable genetic structure. On the other hand, during all the studies carried out on the HP-1967 strain, no modifications in its restriction patters different that those expected related to the genomic deletions have been detected.

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?

Yes ☒	No ☐
If yes, insert the country code(s): The Netherlands (NL)	

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?

Yes ☐	No ☒
If yes: - Member State of notification - Notification number	

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?

Yes ☐	No ☒
If yes: - Member State of notification - Notification number	

7. Summary of the potential environmental impact of the release of the GMOs.

No environmental impact attributable to the GMO release is expected to occur for the following reasons:

The *Actinobacillus pleuropneumoniae* strains are generally very host-specific and not reported to affect human being or other species different from pigs.

The *Actinobacillus pleuropneumoniae* strains are also very sensitive to warm temperatures, sunlight and disinfectants.

The *Actinobacillus pleuropneumoniae* strain HP-1967 has demonstrated not to spread from inoculated to non-inoculated animals.

In the unexpected case of genomic recombination with field strains the strain HP-1967 would recover part of the deleted genomic material. The result obtained after recombination would not be different from a current *Actinobacillus pleuropneumoniae* field strain.

B. Information relating to the recipient or parental organism from which the GMO is derived

1. Recipient or parental organism characterisation:

(a) Indicate whether the recipient or parental organism is a:

(a) Indicate whether the recipient or parental organism is a :	
Viroid	☐
RNA virus	☐
DNA virus	☐
Bacterium	☒
Fungus	☐
Animal	☐
- mammals	☐
- insect	☐
- fish	☐
- other animal	☐ specify phylum, class
Other, specify	

2. Name

(i) order and/or higher taxon (for animals)
(ii) genus Actinobacillus
(iii) species *Actinobacillus pleuropneumoniae*
(iv) subspecies
(v) Strain HP-1967
(vi) pathovar (biotype, ecotype, race, etc.) Serotype 1
(vii) common name *Actinobacillus pleuropneumoniae*

3. Geographical distribution of the organism

(a) Indigenous to, or otherwise established in, the country where the notification is made: Yes ☒ No ☐ Not known ☐
(b) Indigenous to, or otherwise established in, other EC countries (i) Yes ☒ If yes, indicate the type of ecosystem in which is found: Atlantic ☒ Mediterraean ☒ Boreal ☒ Alpine ☒ Continental ☒ Macaronesian ☒ (ii) No ☐ (iii) Not known
(c) Is it frequently used in the country where the notification is made? Yes ☒ No ☐
(d) Is it frequently kept in the country where the notification is made? Yes ☒ No ☐

4. Natural habitat of the organism

(a) If the organism is a microorganism water ☐ Soil, free-living ☐ Soil in association with plant-root systems ☐ In association with plant leaf/stem systems ☐ In association with animals ☒ (Porcine) Other, specify
(b) If the microorganism is an animal: natural habitat or usual agroecosystem:

5(a) Detection techniques

Primary isolation in culture media, biochemical characterisation, serotypification and PCR.

5(b) Identification techniques

Primary isolation in culture media, biochemical characterisation, serotypification and PCR.

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?

Yes ☐	No ☒
If yes, specify	

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extra cellular products), either living or dead?

Yes ☒	No ☐	Not known ☐
If yes: (a) to which of the following organisms: humans ☐ animals ☒ (Porcine) plants ☐ other ☐		
(b) give the relevant information specified under Annex IIIA, point II. (A)(11)(d) of Directive 2001/18/EC The pathogenesis of the Porcine Pleuropneumonia is characterised by three stages: colonization, evasion of the host defense mechanisms and lesion of the target tissues. Colonisation consists of the adhesion capability of the pathogen to the target cells or tissues and multiplication in the host organism. Colonisation is a necessary requisite for the disease development. It has been observed that *Actinobacillus pleuropneumoniae* does not show optimal adherence to the epithelial tissue recovering the trachea and bronchi. However the adherence is optimal in the epithelial tissue that covers the terminal bronchi and alveoli. Once adherence to the target tissues is achieved, the establishment of the infections is conditioned by the bacterium capability of obtaining the necessary nutrients for its propagation. Disponibility of essential nutrients in the respiratory tract is, generally, limited, for that reason the mechanisms intended to obtain the necessary nutrients are considered as pathogenic mechanisms. The mechanism to obtain iron is of the most relevance.		

8. Information concerning reproduction

(a) Generation time in natural ecosystems: 21-28 days
(b) Generation time in the ecosystem where the release will take place: 21/28 days
(c) Way of reproduction Sexual ☐ Asexual ☒
(d) Factors affecting reproduction: Not applicable.

9. Survivability

(a) ability to form structures enhancing survival or dormancy: (i) endospores ☐ (ii) cysts ☐ (iii) sclerotia ☐ (iv) asexual spores (fungi) ☐ (v) sexual spores (fungi) ☐ (vi) eggs ☐ (vii) pupae ☐ (viii) larvae ☐ (ix) other, specify Not applicable.
(b) relevant factors affecting survivability: Temperature, UVA (sunlight), environmental humidity.

10(a) Ways of dissemination

Airborne. Contact between animals.

10(b) Factors affecting dissemination

Low density of animals.

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)

None.

C. Information relating to the genetic modification

1. Type of the genetic modification

(i) insertion of genetic material	☐
(ii) deletion of genetic material	☒
(iii) base substitution	☐
(iv) cell fusion	☐
(v) other, specify	

2. Intended outcome of the genetic modification

Deletion of the genomic sequences corresponding to the exotoxins ApxI and ApxII.

3(a) Has a vector been used in the process of modification?

Yes ☒	No ☐
If no, go straight to question 5.	

3(b) If yes, is the vector wholly or partially present in the modified organism?

Yes ☒	No ☐
If no, go straight to question 5.	

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector plasmid ☒ bacteriophage ☐ virus ☐ cosmid ☐ transposable element ☐ other, specify
(b) Identity of the vector: pApxIΔH2 and pApxIIΔH2
(c) Host range of the vector: Both vectors have been specifically designed to introduce the intended deletions in the genome of *Actinobacillus pleuropneumoniae*
(d) presence in the vector of sequences giving a selectable or identifiable phenotype Yes ☐ No ☒ Antibiotic resistance ☐ Other, specify Indication of which antibiotic resistance gene is inserted
(e) Constituent fragments of the vector The analysis of the recombinant containing the plasmid pApxIΔH2 shows the presence of two new bands of 1.1 and 4.3 kg and the pre-existent band of 20 kb shows a slight increase of 1 kb aprox. The sizes of the new bands are that expected after the insertion of the hybrid plasmid pApxIΔH2 in the 5' flank region of the segment codifying the second transmembrane helix of gen *apxIA* of the Ap genome. The analysis of the recombinant with the plasmid resolved after a second recombination at the same 5' region where the first one was produced, shows that two bands of lower molecular mass disappear, and a slight decrease in the motility of the previous 21 kb band up to be equal to the parental strain is observed. The analysis of the recombinant containing the plasmid pApxIIΔH2 shows that the 15.7 kb band disappears and the presence of three new bands of 8.2, 7.5 and 0.9 kg can be observed. The sizes of the new bands are that expected after the insertion of the hybrid plasmid pApxIIΔH2 in the 3' flank region of the segment codifying the second transmembrane helix of gen *apxIIA* of the Ap genome. The analysis of the recombinant with the plasmid resolved after a second recombination at the same 3' region where the first one was produced shows the presence on one 15.7 kb band which coincides with that observed in the control strain. Finally, the analysis of the recombinant with the plasmid resolved after a second recombination at the 3' flank region of the segment codifying the second transmembrane helix shows that the 13.5 and 0.9 kb bands disappear and a new 7.5 kb fragment is observed. This band distribution is that expected once the segment codifying the second transmembrane helix disappears and is substituted by one *Eco*RI target. This new target inserted in the App genome divides the 15,7 kb *Eco*RI, which included the operon *apxII*, into to fragments of 8.2 and 7.5 kb respectively.

(f) Method for introducing the vector into the recipient organism (i) transformation ☒ (ii) electroporation ☐ (iii) macroinjection ☐ (iv) microinjection ☐ (v) infection ☐ (vi) other, specify

5. If the answer to question B.3 (a) and (b) is no, what was the method used in the process of modification?

Not applicable.

(i) transformation ☐ (ii) microinjection ☐ (iii) microencapsulation ☐ (iv) macroinjection ☐ (v) other, specify Not applicable.

6. Composition of the insert

Not applicable.

(a) Composition of the insert
(b) Source of each constituent part of the insert
(c) Intended function of each constituent part of the insert in the GMO
(d) Location of the insert in the host organism - on a free plasmid ☐ - integrated in the chromosome ☐ - other, specify
(e) Does the insert contains parts whose product or function are not known Yes ☐ No ☐ If yes, specify

D. Information on the organism(s) from which the insert is derived

Not applicable.

1. Indicate whether it is a:

viroid		☐
RNA virus		☐
DNA virus		☐
bacterium		☐
fungus		☐
animal		☐
- mammal	☐	
- insect	☐	
- fish	☐	
- other animal	☐	(please specify phylum, class)
- other, specify		

2. Complete name

(i) order and/or higher taxon (for animals)
(ii) family name (for plants)
(iii) genus
(iv) species
(v) subspecies
(vi) strain
(vii) cultivar/breeding line
(viii) pathovar
(ix) common name

3. Is the organism significantly pathogenic or harmful in any other way (including its extra cellular products), either living or dead?

Yes ☐	No ☐	Not known ☐
If yes, specify the following (a) to which of the following organisms?	humans ☐ animals ☐ plants ☐ other ☐	
(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism? Yes ☐ No ☐ Not known ☐		
If yes, give the relevant information under Annex IIIA, point II(A)(11)(d):		

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?

Yes ☐	No ☐
If yes, specify	

5. Do the donor and recipient organism exchange genetic material naturally?

Yes ☐	No ☐	Not known ☐

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned? Yes ☒ No ☐ Not known ☐ Specify: the GMO has less survival capacity into the environment
(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned? Yes ☐ No ☒ Not known ☐ Specify
(c) is the GMO different from the recipient as far as dissemination is concerned? Yes ☒ No ☐ Not known ☐ Specify The GMO shows no spread capability from inoculated to non-inoculated animals when compared with the parental strain.
(d) is the GMO different from the recipient as far as pathogenicity is concerned? Yes ☒ No ☐ Not known ☐ Specify The GMO shows a reduced pathogenicity when compared to the parental strain.

2. Genetic stability of the genetically modified organism

The genetic pattern remains stable up to 5 serial passages in culture and does not revert to virulence after 2 serial passages in pigs.

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?

Yes ☐	No ☒	Not known ☐
If yes, specify the following (a) to which of the following organisms?	humans ☐ animals ☐ plants ☐ other ☐	
(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i) The course of a natural outbreak caused by a virulent strain of *Actinobacillus pleuropneumoniae* can be summarised as follows: the pathogen strain colonises the target animal by inhalation and colonises the tonsils, terminal bronchi and alveoli. The pathogen adheres to different factors of the epithelial tissue of the low respiratory tract. The clinical onset originates once the lung defence mechanisms fail; which is due to the Apx exotoxins release, which is responsible for the disease characteristic lesions. The OGM is named *Actinobacillus pleuropneumoniae*, strain HP-1967. This strain is characterised by the introduction of two genomic deletions in one segment of the gen A codifying one transmembrane domain of the ApxI and Apx II exotoxins respectively. The transmembrane domains of the exotoxins ApxI and ApxII have an important role in the pore formation. Both exotoxins shows haemolytic and cytolytic activities, thus the recombinant strains does not have such properties and constitutes an attenuated strain. This gene-deleted strain maintains the non-haemolytic toxins intact and contains all the immunogen antigens necessary to induce a protective immune response. Summing up, *Actinobacillus pleuropneumoniae* strain HP-1967, is characterised to be apathogen for the target species and confers a satisfactory immunity against challenge with virulent strains of *Actinobacillus pleuropneumoniae.*		

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment Culture isolation and PCR.
(b) Techniques used to identify the GMO Culture isolation and PCR.

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)

Assessment of the safety and efficacy of this GMO as vaccine strain against Porcine Pleuropneumonia.

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?

Yes ☐	No ☒
If yes, specify	

3. Information concerning the release and the surrounding area

(a) Geographical location (administrative region and where appropriate grid reference): Farms sited in the provinces of Lleida, Toledo, Segovia and Valladolid (Spain).
(b) Size of the site (m^2): (i) actual release site ($m^{2)}$: 165 Ha. approx. (ii) wider release area (m^2): 165 Ha. approx.
(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected: None.
(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO Growing of cereals, vineyards and fruit trees. Fauna: rabbits, birds, foxes and wild boars.

4. Method and amount of release

(a) Quantities of GMOs to be released: Vaccinated animals will receive 2 minimum doses of 2 x 10^8 cfu/dose. The GMO will be administered by intramuscular injection to pigs.
(b) Duration of the operation: The GMO will be released 2 days (vaccination and revaccination days). The observation period will last for 6 months.
(c) Methods and procedures to avoid and/or minimize the spread of the GMOs beyond the site of the release No spread of the GMO is expected to occur, as it will be inoculated by intramuscular injection and the GMO has demonstrated not to spread from inoculated animals. In any case the animals will be housed in isolated farms.

5. Short description of average environmental conditions (weather, temperature, etc.)

The weather in Catalonia is Mediterranean. The summer season is very hot and winter is not so cold. The rainfall rate is scarce and irregular and use to concentrate in spring and autumn Cataluña. In some cases heavy rainstorms and floods occur quite often. There are a lot of sunny days per year. The weather in Castilla-León is Continental. Temperatures are characterised to be extreme, very cold and long winters and hot and short summers. Rainfalls are very scarce and concentrated in spring and winter. Intermediate seasons hardly appear.

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.

None.

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

1. Name of target organism (if applicable)

(i) order and/or higher taxon (for animals) *Vertebrae*
(ii) family name (for plants)
(iii) genus *Suis*
(iv) species *Sus scrofa*
(v) subspecies
(vi) strain
(vii) cultivar/breeding line
(viii) pathovar
ix) common name Porcine (fattening pigs).

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)

Replication of the GMO in the inoculated animal, without producing adverse reactions and inducing active immunity against Porcine Pleuropneumonia.

3. Any other potentially significant interactions with other organisms in the environment

None.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes ☐	No ☒	Not known ☐
Give details		

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established

None.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO

None.

(i) order and/or higher taxon (for animals)
(ii) family name (for plants)
(iii) genus
(iv) species
(v) subspecies
(vi) strain
(vii) cultivar/breeding line
(viii) pathovar
ix) common name

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem: Recombination between the GMO and field strains is unlikely to occur. In such a case, the GMO would incorporate part of the deleted genome sequences. This fact is not considered to have any negative impact for the environment.
(b) from other organisms to the GMO: None.
(c) likely consequences of gene transfer: None.

8. Give references to relevant results (if available) from studies of the behavior and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):

Different assays using the gene-deleted strain HP-1967 have demonstrated that such strain is less pathogenic than the parental one, does not interfere with the environment as it does not spread from inoculated animals, and are able to protect pigs against the disease.

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)

None.

H. Information relating to monitoring

1. Methods for monitoring the GMOs

Detection of anti-gE antibodies in the inoculated and non-inoculated animals. Physical barriers in the release area. Absence of GMO sensitive wildlife.

2. Methods for monitoring ecosystem effects

Not applicable, as no effects on the environment are to be produced. The presence of the GMO in the wild fauna can be verified if considered necessary.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms

Not applicable.

4. Size of the monitoring area (m^2)

165 Ha. approx.

5. Duration of the monitoring

Six months (duration of the whole trial).

6. Frequency of the monitoring

Daily.

I. Information on post-release and waste treatment

1. Post-release treatment of the site

None, as GMO release into the environment is not expected to occur.

2. Post-release treatment of the GMOs

None.

3(a) Type and amount of waste generated

Glass vials containing the freeze-dried GMO, and plastic materials for inoculation and sample collection.

3(b) Treatment of waste

Glass vials, syringes, needles, tubes and other materials in contact with the GMO will be sterilized by incineration or autoclave.

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread

Sacrifice and incineration of all the animals of the farm and disinfection of all the facilities.

2. Methods for removal of the GMO(s) of the areas potentially affected

Formaldehyde, phenols and UV.

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread

All the animals will be sacrificed and incinerated immediately.

4. Plans for protecting human health and the environment in the event of an undesirable effect

The GMO is based on the *Actinobacillus pleuropneumoniae* bacterium, which it is reported not to affect human beings, other animal species or plants.