P12

Optimization of the detection of Clostridium botulinum in pig and cattle manures and in digestates from on-farm biogas plants

Le Marechal C.[1], Rouxel S.[1], Poëzevara T.[1], Druilhe C.[2], Pourcher A.-M.[3], Denis M.[1]

[1]Anses - Laboratoire de Ploufragan, Ploufragan, France, [2]IRSTEA, Ploufragan, France, [3]IRSTEA, Rennes, France

Introduction

Anaerobic digestion (AD) is a sustainable technology for converting livestock manure into biogas. A raise in the number of agricultural biogas plants (BGP) has been observed recently in several European countries. The fate of pathogens, in particular *Clostridium botulinum* (Cb) during AD and the sanitary risks through spreading on land appears to be a matter of public health concern. Asymptomatic carriage of Cb has in fact been demonstrated in fecal contents of cattle and pigs [1, 2]. Clostridia being spore-forming anaerobic bacteria, their ability to form spores confers them a high resistance to environmental conditions, while their ability to grow under mesophilic anaerobic conditions raises the question of their future and the potential for their multiplication during AD. Proliferation and environmental contamination through digestate spreading on lands when using manures that may contain Cb for AD have been hypothesized [3, 4]. A first study conducted to address this topic using laboratory-scale digester invalidated this hypothesis [5]. Further studies including field investigations are now required.

Manure and digestate are complex matrices with rich microflora. The detection and enumeration of pathogens in such matrices can be challenging, especially in the absence of selective media and when the level of the pathogen is low or close to the limit of detection of the method. No prescriptive or consensual method is available for the detection of Cb in such matrices. It is thus necessary to adapt protocols developed for food or clinical samples to maximize the detection of target pathogens.

The objective was to optimize the detection of Cb in manure and digestate samples using naturally contaminated samples collected in agricultural BGP.

Material and Methods

Samples

Manure and digestate were collected from five biogas plants (BGP1 to BGP5) located in France. The livestock effluents to be treated through AD were either pig manure (BGP1, 3 and 4), cattle manure (BGP2) or both (BGP5). Each BGP was visited once. The manure and digestate of each BGP were collected in three replicates, transported at room temperature for less than one hour, and analyzed on the same day.

Detection of *C. botulinum*

For detection, two methods (M1 and M2) and six protocols (P1 to P6) were used (Fig. 1) regardless of the form (vegetative or spore cells). For the first method (M1), 25 g of each sample were 10-fold diluted in pre-reduced Trypticase Peptone Glucose Yeast broth (TPGY) and homogenized using a Pulsifier (Microgen, Surrey, UK) for 15 seconds. For the second method (M2), 10 g of each sample were 10-fold diluted in pre-reduced TPGY, incubated for 10 minutes at 70°C in a water bath, and cooled for one minute in cold water.

The samples were then incubated at 37°C in an anaerobic chamber (A35, Don Whitley) filled with anaerobic gas (10% H_2, 10% CO_2, 80% N_2). After 24 hours (P1 and P4), four days (P2 and P5) and 10 days (P3 and P6) of incubation, 1 ml was collected for DNA extraction.

DNA extraction was performed using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel) according to the manufacturer's instructions.

Detection of the encoding genes for BoNT types A, B, E and F and a group III target was performed using real-time PCR with a Bio-Rad CFX96 thermal cycler as previously described [6, 7]. A sample was considered positive when a characteristic amplification was detected.

Results

Six protocols were compared for the detection of Cb (Fig. 1), with or without thermal treatment at 70°C, and with different incubation periods (24 hours, 4 days and 10 days). The highest detection level (16 positive samples out of 30) was obtained using the P1 protocol (Fig. 1) by analyzing 25 g samples 10-fold diluted in TPGY without thermal treatment, with 24 hours of incubation at 37°C in an anaerobic chamber. Cb was detected in all BGP except in BP1. The most common gene (present in 100% of the positive samples) was that encoding BoNT type B.

Discussion and Conclusion

Several protocols were tested here on naturally contaminated manure and digestate samples to select the most suitable one to be able to detect Cb. A short incubation period was selected for the detection of Cb in manure and digestate on the contrary to some previous studies studying environmental samples [8, 9]. Optimal incubation period of only 18 hours for the detection of Cb in pig fecal samples was already observed [1]. This protocol is now available to evaluate the fate of Cb during AD.

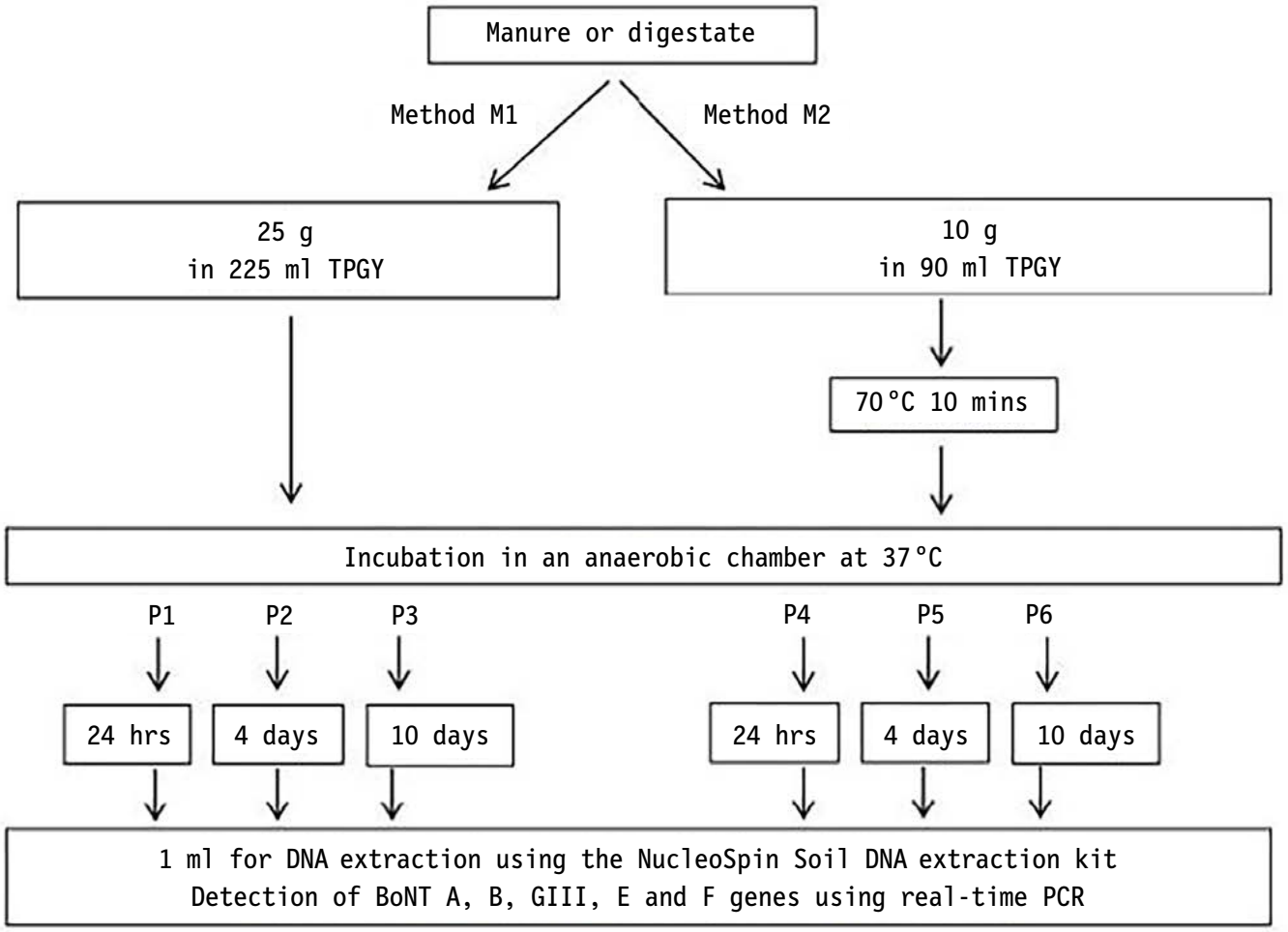

Figure 1: Sample analysis workflow for the detection of C. botulinum for manure and digestate

References

1. Dahlenborg M, et al.: Development of a Combined Selection and Enrichment PCR Procedure for *Clostridium botulinum* Types B, E, and F and Its Use to Determine Prevalence in Fecal Samples from Slaughtered Pigs. Applied and Environmental Microbiology 2001, 67(10):4781-4788.
2. Dahlenborg M, et al.: Prevalence of *Clostridium botulinum* types B, E and F in faecal samples from Swedish cattle. International Journal of Food Microbiology 2003, 82(2):105-110.
3. Botulinumtoxikosen - Infektionsrisiken für Mensch und Tier (unter besonderer Berücksichtigung von Schwein bzw. Rind). Nutztierpraxis aktuell 33, 14-18.http://www.botulismus.org/index_htm_files/botulinumtoxikosen%2003032010.pdf (accessed 19 April 2019).
4. Boll C: Chronischer Botulismus - Tod aus der Biogasanlage. Wild und Hund 2011(10):14-19.
5. Froschle B, et al.: Fate of *Clostridium botulinum* and incidence of pathogenic clostridia in biogas processes. J Appl Microbiol 2015, 119(4):936-947.
6. Fach P, et al.: Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. J Appl Microbiol 2009, 107(2):465-473.
7. Woudstra C, et al.: Molecular gene profiling of *Clostridium botulinum* group III and their detection in naturally contaminated samples originating from various European countries. Appl Environ Microbiol 2015.
8. Neuhaus J, et al.: Detection of *Clostridium botulinum* in liquid manure and biogas plant wastes. Folia microbiologica 2015.
9. Vidal D, et al.: Real-time polymerase chain reaction for the detection of toxigenic *Clostridium botulinum* type C1 in waterbird and sediment samples: Comparison with other PCR techniques. Journal of Veterinary Diagnostic Investigation 2011, 23(5):942-946.