Original Research Article

Identification of *Nosema Sp*. *Paenibacillus larvae* and *Melissococcus plutonius* by PCR in Benin's apiaries.

ABSTRACT

The bee A. mellifera L. is a key social insect, for its role in crop pollination and for the products generated in the hives. *Nosema sp. Paenibacillus larvae and Melissococcus plutonius belong to the most important pests of A. mellifera Linnaeus* bees *(Hymenoptera: Apidae).* The aim of this study was to identify the presence of *Nosema Sp., Paenibacillus* larvae and *Melissococcus plutonius* in Benin's apiaries by molecular characterization. Thirty-seven bee samples from three districts of Benin (North-East, North-West and Centre) were analysed in the study. We used Polymerase Chain Reaction (PCR) tests based on 16 S ribosomal RNA and primers specific to each of the parasites of interest to identify them in the bee samples. Data were analyzed with graphpad prisms 8 and minitab 19. Only *N. ceranae* belonging to *Nosema Sp* was found in the tested samples with a rate of 13.51%. This indicates that the nosema parasite is present in the apiaries of Benin. This is the first report of *N. ceranae* in *A. mellifera* colonies in Benin. Intensive surveys should be carried out to determine the distribution and prevalence of *N. ceranae* in the different regions of Benin in order to effectively control this parasite.

Keywords: Apis mellifera, Melissococcus plutonius, Nosema Sp, Paenibacillus larvae, Polymerase Chain Reaction, Benin.

1. INTRODUCTION

The honey bee has been exploited by humans in beekeeping for about 7000 years [1]. The interest in this bee is due to its ability to produce honey, royal jelly, wax and also to harvest pollen and propolis [1-2]. In addition, they contribute more than 80% of the pollination services to global agriculture [3-4], which have been estimated to be worth 153 billion euros per year [5-6]. Bees are involved in the pollination of crop plants such as fruit trees, vegetables and fodder, as well as plants that may be involved in the manufacture of biofuels, and thus ensure the maintenance of plant biodiversity [6,7]. Thus, any threat to pollinating bees, whether from pesticides, herbicides or disease, has serious consequences not only for beekeeping but also for agriculture in general [7].

Precise data on the causes of bee mortality in Benin are often not known, but there are a number of factors that threaten the local bee and negatively influence the production of hive products; a previous field survey of beekeepers in Benin [8] highlighted the main role of bee pathogens, in particular *Varroa* and intoxication of bees by insecticide treatments, as well as a degradation of the ecosystem (reduction of the melliferous flora) with the influence of climate change. Also, in certain localities, a decrease in the health of the population and the yield of the colony has been observed, with tired bees and even the disappearance of certain colonies. It has also been noted that waste and excrement inside some hives and around the entrance are tell-tale signs of Nosema and foulbrood. Nosema is generally considered to be one of the most destructive diseases of adult bees, affecting workers, queens and also males. It is a disease that affects the digestive tract causing acute

diarrhoea [9] and can in some cases lead to high mortality of affected colonies. The disease is caused by microsporidia, a group of obligate intracellular parasites related to fungi (Nosema sp.), [10]. The parasite forms resistant spores that remain viable for long periods of time. It results in tremors in bee imagos, inability to fly and a decline of the colony until it disappears [11]. Nosema exists in two species: *N. apis,* ubiquitous, a parasite of the European honey bee, *Apis mellifera*, an obligate intracellular parasite, which cycles within the host cell and occurs in two forms: the spore, the infective agent and the vegetative form. *N. ceranae* is a parasite of the Asian honey bee, *Apis cerana* and of *A. mellifera*. *Nosema ceranae* has been detected in different geographically separated populations of *Apis mellifera* in Europe, South and North America and Asia [10, 12]. Bees can die within 8 days of exposure to *Nosema ceranae*. The pathogenic effects of *N. ceranae* on *Apis mellifera* colonies are not fully known. *N. ceranae is* thought to be involved in the weakening of bee colonies in the presence of other stress factors.

The microspore parasite *Nosema apis* infects the epithelial cells of the ventricles of the honey bee (*Apis mellifera*) and is widespread worldwide [13] but so far is not considered a significant problem in tropical and subtropical climates [13]. Historically, *Nosema apis* (*N. apis*) was known to be a well-established pathogen of *A. mellifera* [14]. Over the last decade, *N. ceranae has been* frequently reported to infect honey bees [14]. It is now thought that both *N. apis* and *N. ceranae* are widespread worldwide and honeybees are also co-infected with both species. Field diagnostic methods are based on observation of symptoms in the colony but the spores produced by the two Nosema species are very similar in shape and can only be differentiated with difficulty by the conventional light microscopy method. It is necessary to use molecular biology techniques to identify infections or co-infections by each of these pathogens [15]. PCR can be used to distinguish between *Nosema apis* and *Nosema ceranae*. The general objective of this study is to investigate the presence and type of Nosema in beehives in Benin in order to effectively control this parasite.

2. MATERIAL AND METHODS

2.1 Collection Sites

The collection of bee samples was carried out in the three agro-ecological zones of Benin. These are the central zone (zone 1), the north-eastern zone (zone 2) and the north-western zone (zone 3) in Benin [16] (figure 1).

The Benin (114,763 km²) is a country in West Africa situated between meridians 0°40' and 3° 45' east longitude and parallels 06 ° 15 'and 12 ° 25' north latitude. The Collines Department (Zone 1) is wholly owned by the Guinean Sudanese climate zone marked by two rainy seasons that cover the periods from April to July and October to November. It is a transition zone (between South and North) of 16,900 km² extending after the plates of Abomey and Kétou until 9th parallel north. This area is fully occupied by leached tropical ferruginous soils or depleted [17]. One also meets vertisols and waterlogged soils in the valleys of rivers and streams that cross the area. Zones 2 and 3, being in the Sudanese agro-ecological area, are between the 9th and le10e parallel north and a little beyond. They cover some of Atacora and Donga. North East (Zone 2) is characterized by a Sudanese climate with a rainfall from April to October and a season dry season from November to March. Northwest of Benin (Zone 3) essentially has a mountain climate and has slight variations from one locality to another. Zones 2 and 3 are mainly dominated by tropical ferruginous soils with highly variable agronomic characteristics. These soils fine clay-sandy texture. We also meet lateritic soils and waterlogged soils in these areas [17]. Benin's population is estimated at 9,983,884 inhabitants with a density of 57 inhabitants / km² [18]. The main activities of the local population are agriculture, livestock and fisheries. Some

farmers in Benin associate beekeeping agriculture for a better performance given the role of bees in the pollination of crops. Nowadays, beekeeping of Benin is facing many problems.

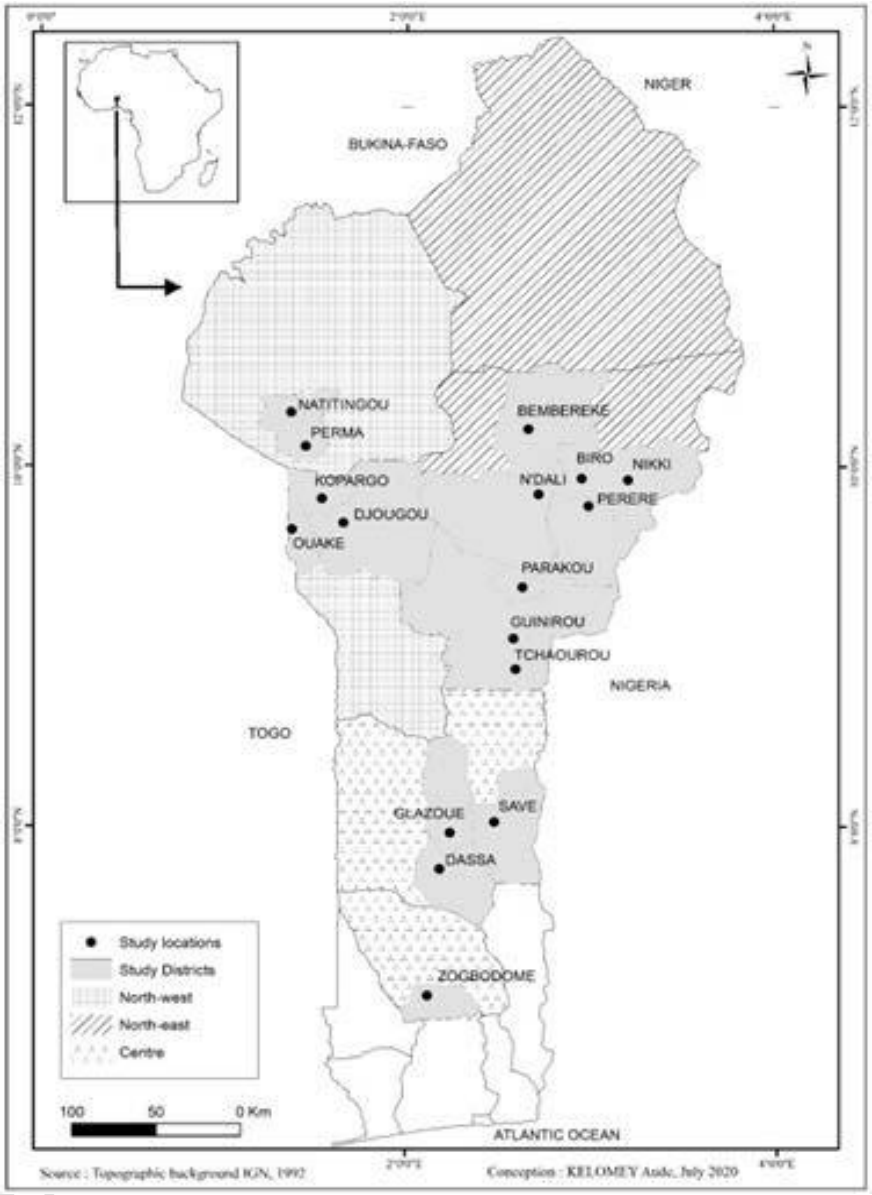

Figure 1: Map showing the area of sample collection in the three zones

2.2. Samples collection

Municipalities were selected in each zone according to the abundance of beekeeping. These areas were chosen based on criteria parasite infestation rate and availability of beekeeping equipment. By town one or two localities have chosen. A total of 17 municipalities were selected in the study (Figure 1). A total of 37 bee colonies (per one bee colony) were collected in the three agro-ecological zones, including 11 colonies in the northwest, 08 colonies in the centre and 18 colonies in the northeast. Before capturing the bees, the flight

holes of the selected colonies were smoked to calm them down and make them less aggressive. The bees were captured with fine tweezers and kept in an Eppendorf tube containing 2 ml of “RNA later” solution and stored at room temperature for one (1) week and at -20°C. The samples were transported to the Evolution Genome Behaviour and Ecology Laboratory of the Centre National de la Recherche Scientifique (CNRS) of France at its site of Gif-sur-Yvette for molecular analysis to identify Nosema parasites.

2.3. Samples treatment

2.3.1. DNA extraction

DNA extraction was performed by grinding the 10 bees from each colony, kept on ice, in a phosphate buffer solution using a mechanical grinder (1mL of PB for 1 bee). The supernatant was recovered under PSM after centrifugation at 4°C. The operation was repeated once, and the grindings were stored at -20°C.

Next, we used the simplified centrifugal DNA extraction protocol with the QIAGEN kit (51306), the QIAamp DNA Mini kit (250) to extract DNA from grindings [19] (Bourgeois *et al.* 2010). This is a DNA extraction method with proteinase K, buffer solutions and ethanol.

2.3.2. PCR design methodology

Bacteria are identified in the study by amplification of 16S ribosomal RNA DNAs according to the protocol of Chen *et al.* [20]. The DNAs are amplified by PCR using primers specific to each parasite sought in the study. The primer sequences used are described in Table 1. The technique used is based on the PCR protocol described by Dobbelaere *et al*. [21]. The technique allows *in vitro* amplification of a specific region of a given nucleic acid. PCR was performed using a thermocycler (Biometra) in 25 µL volumes containing 9 µL H2O, 10µL of Master mix (green taq thermofisher Master mix containing:Tp Taq Pol 10X, MgCl2, 50 mM, dream Taq Pol 5 u/µL, dNTP 10 mM,0.5 µL Primer 1 and Primer 2 (20 µM) and 5 µL DNA. We used 4 Mixes, 2 for (*Nosema ceranea* and Apis), 1 for *M. plutonius*, and 1 for *P. larvae.* The thermal cycler program consisted of 94 °C for 15 seconds followed by 25 cycles of 15 s at 94 °C, 30 s at 62 °C and 45 s at 72 °C and a final extension step at 72 °C for 7 min for *Nosema ceranea and Apis.* The thermal cycler program consisted of 94 °C for 2min followed by 30 cycles of 30s at 94 °C, 30s at 55 °C and 1 min at 72 °C and a final extension step at 72 °C for 5min for *P. larvae* (AFB 3 and AFB 4) and *M. plutonius* (EFB 1 and EFB 2). The amplified PCR products were subjected to electrophoresis through the 1.6% TBE agarose gel in standard TBE buffer, stained with ethidium bromide and visualised on a UV table. The specific primers of *Nosema Sp.* (apis and ceranae), *P. larvae* (AFB 3 and AFB 4) and *M. plutonius* (EFB 1 and EFB 2) are stored at -20 °C.

Table 1: Sequences of primers for *Nosema Sp.*, *M. plutonius* and *P. larvae.*

Primers	Sequences	PCR product size	Specificity
218MITOC-FOR	5'-CGGCGACGATGTGATATGAAAATATTAA-3'	218-219 Pb	*N. ceranae*
218MITOC-REV	5 - 'CCCGGTCATTCTCAAACAAAAAACCG-3'		
321APIS-FOR	5'-GGGGGCATGTCTTTGACGTACTATGTA-3'	321 Pb	*N. apis*

321APIS-REV	5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'		
EFB1	5-'GAA GAG GAG TTA AAA GGC GC-3'	832 Pb	*M. plutonius*
EFB2	5'-TTA TCT CTA AGG CGT TCA AAG G-3'		
AFB3	5'-CTT GTG TTT CTT TCG GGA GAC GCC A-3'	1096 Pb	*P. larvae*
AFB4	5'-TCT TAG AGT GCC CTC TGC G-3'		

2.4. Statistical analysis of data

The collected data were processed with Excel 2016 and then analyzed with GraphPad Prisms 8, R and Minitab 19. P values < 0.05 are considered significant.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. Variability of fragment lengths

The DNA fragments obtained on the agarose gel revealed that the positive samples obtained were all 218 bp in amplicon size (Figure 2). This indicates that the positive samples are not polymorphic and are all positive for the *Nosema ceranae* parasite.

In the 37 bee colonies analyzed, no presence of the parasite *Nosema apis* was observed (0% occurrence rate). On the other hand, *Nosema ceranae* is present in the populations studied (occurrence rate 13.5%). The frequency of distribution of this parasite across the three study areas is presented in figure 2.

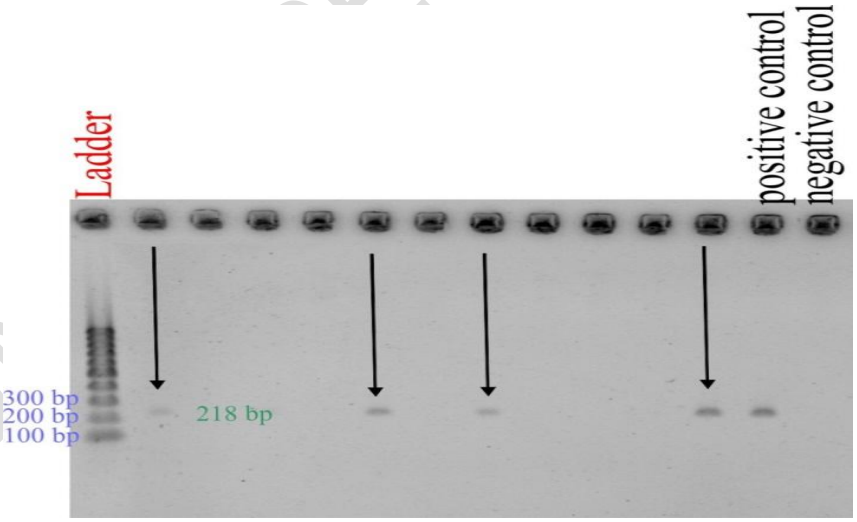

Figure 2: Positive samples on 1.6% agarose gel. 100bp DNA ladder de Jena Bioscience.

3.1.2. Occurrence of pests in the study areas

The results of the binary logistic regression on the occurrence of parasites in terms of presence and absence in the apiaries of bees from different production zones in Benin revealed that the presence of the parasite species studied did not depend at the 5% threshold on the production zone, nor on the type of parasite species (Table 2).

Table 2. Results of the binary logistic regression

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-41,6	4730	-0,009	0,993
The_European	0	3440	0	1
N_Apis	0	3440	0	1
N_Ceranae	20,8	2430	0,009	0,993
COP	0	57.40	0	1
DAS	0	6410	0	1
DJO	20,8	4060	0,005	0,996
GLA	0	6410	0	1
NAT	19,7	4060	0,005	0,996
NOA	0	5740	0	1
NIK	0	6410	0	1
OUA	0	6410	0	1
BY	20,1	4060	0,005	0,996
PER	20,80	4060	0,005	0,996
AFTER-SALES SERVICE	0	6410	0	1
TCH	19,4	4060	0,005	0,996
ZOG	0	6410	0	1

The analysis of Table 3 shows specifically that *Nosema Ceranae* alone was present in the apiaries and mainly in the zones of DJO, NAT, PER, PAR, and TCH. *Nosema Ceranae* was absent in the center but present in the northwest and northeast with an infestation intensity of 40 and 50% respectively (figure 3).

Table 3. Occurrence of parasites by study area

Sites	The_American	The_Europea n	N_Apis	N_Cerana e
BEM	0	0	0	0
COP	0	0	0	0
DAS	0	0	0	0
DJO	0	0	0	1
GLA	0	0	0	0
NAT	0	0	0	1

NOA	0	0	0	0
NIK	0	0	0	0
OUA	0	0	0	0
BY	0	0	0	1
PER	0	0	0	1
AFTER- SALES SERVICE	0	0	0	0
TCH	0	0	0	1
ZOG	0	0	0	0

Legend: 1= presence, 0 = absence

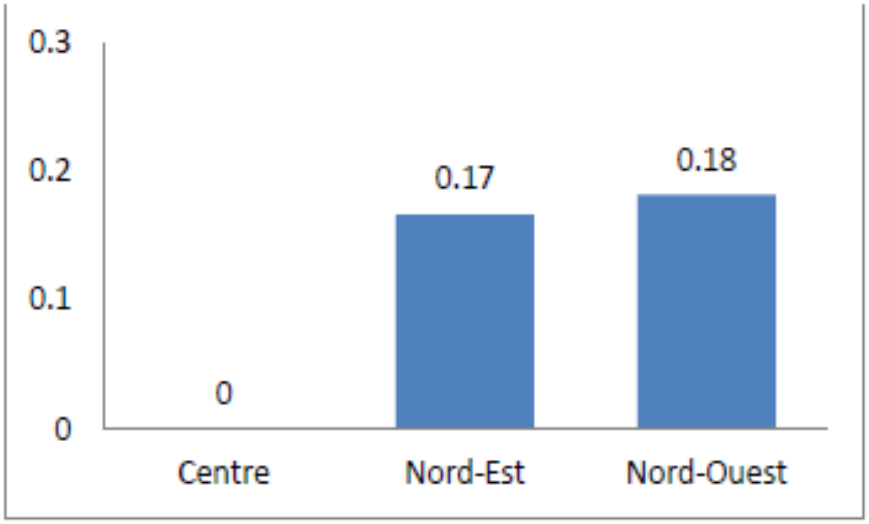

Fig 3: Frequency of distribution of *Nosema ceranae* in bee colonies

A comparison test of two proportions was performed on the proportions observed in the North-East and North-West zones. The results showed that there was no significant difference between the proportions 3/18 and 2/11 observed in these two northern zones for *Nosema ceranae*.

Table 4: Chi_2 test of independence

	Centre	Nord-Ouest	Nord-Est	Significativité
N.Ceranae	0	3	2	NS
N.apis	0	0	0	

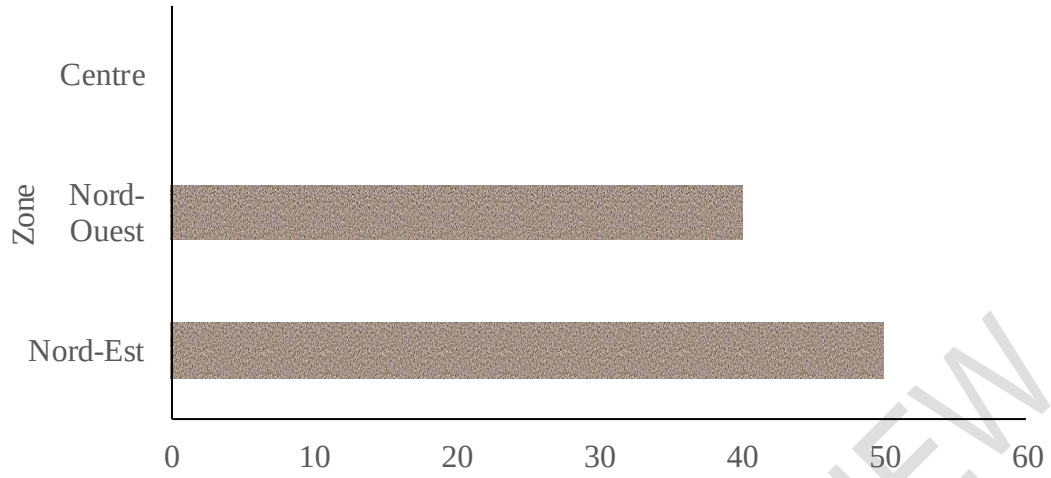

Fig 4. Degree of infestation of *Nosema Ceranae* by production area

3.1.3. Frequency of occurrence of *Nosema Ceranae*

Analysis of this table reveals that *N. ceranae* appears in 5 zones out of 37, i.e., 13%. The presence of this parasite depends on the 5% threshold of the production area.

Table Binomial with n = 1 and p = 0.027

Chart 1 : Frequency of occurrence of Nosema Ceranae

Zone	x	P(X ≤ x)
COP	0	0.973
TCH	0	0.973
GLA	0	0.973
NIK	0	0.973
BY	0	0.973
BEM	0	0.973
TCH	1	**1.000**
NOA	0	0.973
BY	1	1.000
ZOG	0	0.973
DJO	1	1.000
OUA	0	0.973
DAS	0	0.973
NAT	1	1.000
PER	0	0.973
COP	0	0.973
TCH	0	0.973
COP	0	0.973
DJO	0	0.973

OUA	0	0.973
TCH	0	0.973
GLA	0	0.973
NAT	0	0.973
ZOG	0	0.973
DAS	0	0.973
NOA	0	0.973
TCH	0	0.973
NAT	0	0.973
BY	0	0.973
BEM	0	0.973
BEM	0	0.973
PER	1	1.000
NIK	0	0.973
NAT	0	0.973
NOA	0	0.973

Analysis of this table reveals that *N. ceranae* appears in 5 zones out of 37, i.e. 13%. The presence of this parasite depends on the 5% threshold of the production area.

3.1.1. Frequency of occurrence of *Nosema apis*

Nosema apis does not occur in any area. The frequency of occurrence of this parasite is not related to the study area.

Chart 2 : Frequency of occurrence of Nosema apis

Zone	**x**	**P(X ≤ x)**
COP	0	0.973
TCH	0	0.973
GLA	0	0.973
NIK	0	0.973
BY	0	0.973
BEM	0	0.973
TCH	0	0.973
NOA	0	0.973
BY	0	0.973
ZOG	0	0.973
DJO	0	0.973
OUA	0	0.973
DAS	0	0.973
NAT	0	0.973
PER	0	0.973
COP	0	0.973
TCH	0	0.973

COP	0	0.973
DJO	0	0.973
OUA	0	0.973
TCH	0	0.973
GLA	0	0.973
NAT	0	0.973
ZOG	0	0.973
DAS	0	0.973
NOA	0	0.973
TCH	0	0.973
NAT	0	0.973
BY	0	0.973
BEM	0	0.973
BEM	0	0.973
PER	0	0.973
NIK	0	0.973
NAT	0	0.973
NOA	0	0.973

3.1.1. Parasitic foulbrood: American foulbrood and European foulbrood

The PCR tests carried out revealed no presence of these two parasites in the 37 bee colonies investigated.

3.2. Discussion

The objective of this study was to investigate the presence of the parasites *Nosema Sp.*, *Paenibacillus larvae* and *Melissococcus plutonius* in apiaries in Benin. The results of the binary logistic regression on the occurrence of parasites in terms of presence and absence in the sampled beehives revealed that the presence of the studied parasite species did not depend at the 5% threshold on the production area, nor on the type of parasite species (Table 2). These results show that the type of parasite does not depend on the study area and indicate that the parasites investigated, if present in the bee colonies, could be found in all regions covered by the study area. The results obtained corroborate the findings of Chauzat *et al.* [22] which reported the presence of a large number of parasites (*Aetina timuda*, *Varroa destructor*, *Nosema Sp*., *Paenibacillus larvae* and *Melissococcus plutonius*) in all the apiaries in tested their studies.

The analysis of table 3 shows that *Nosema Ceranae* alone was found in the apiaries and mainly in the zones of DJO, NAT, PER, PAR, and TCH. This result shows that the other parasites searched for (*Paenibacillus larvae* and *Melissococcus plutonius*) are not yet found in the apiaries of Benin or have not been identified in the sample collections made in this study. *Nosema ceranae* was absent in the center but present in the North-West and North-East with an infestation intensity of 40 and 50% respectively, indicating that the *Nosema ceranae* parasite has not yet colonized the apiaries in the center of Benin given the infestation rate in the two northern parts of the country.

No presence of the parasite *Nosema apis* was observed (0% occurrence rate). This could be explained by the fact that since the emergence of N*. ceranae as a* new pathogen of *A.*

Mellifera, *N. ceranae* is found all over the world [14] and also in Africa in contrast to *Nosema apis* which is widespread worldwide [13] but so far not considered as a major problem in tropical and subtropical climates [13].

4. CONCLUSION

The presence of the parasites *Nosema Sp., Paenibacillus larvae* and *Melissococcus plutonius* creates important economic losses in the beekeeping sector. The results show that the parasite identified in the analysed samples belongs to the *Nosema ceranae* subspecies *of Nosema Spp*. We did not identify the *Nosema apis* subspecies nor the parasites *Paenibacillus larvae* and *Melissococcus plutonius* in our sample. However, the analysis of a large number of samples in a larger number of apiaries in the three study areas remains a means to determine more precisely the percentage and types of parasites in apiaries in Benin.

COMPETING INTERESTS DISCLAIMER:

AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. THE PRODUCTS USED FOR THIS RESEARCH ARE COMMONLY AND PREDOMINANTLY USE PRODUCTS IN OUR AREA OF RESEARCH AND COUNTRY. THERE IS ABSOLUTELY NO CONFLICT OF INTEREST BETWEEN THE AUTHORS AND PRODUCERS OF THE PRODUCTS BECAUSE WE DO NOT INTEND TO USE THESE PRODUCTS AS AN AVENUE FOR ANY LITIGATION BUT FOR THE ADVANCEMENT OF KNOWLEDGE. ALSO, THE RESEARCH WAS NOT FUNDED BY THE PRODUCING COMPANY RATHER IT WAS FUNDED BY PERSONAL EFFORTS OF THE AUTHORS.

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