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Immunological quantification of the nematode parasitic bacterium *Pasteuria penetrans* in soil

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Abstract

Currently, the abundance of *Pasteuria penetrans* in soils, an unculturable bacterial parasite of root-knot nematodes (*Meloidogyne* spp.), is estimated by the percentage of nematode juveniles infected with bacteria and the number of spores attached to their cuticle. Indirect immunofluorescence led to detection of free spores directly in soil suspensions using UV light and polyclonal antibodies raised against two *P. penetrans* populations (ORS-21414-Sen and PP1). Three extraction methods were compared in order to improve spore recovery. A gentle shaking/sieving method recovered more than 90% of the spores inoculated in soils and was more efficient and simple than aqueous two-phase partitioning and polyethylene glycol extractions. All the spores inoculated in sandy or sandy–clay soils were detected with immunofluorescence microscopy. The quantification of the spores was improved using an ELISA technique that showed a good correlation between optical density and spore concentration in inoculated soils. Specific antibodies provide a suitable method to quantify *P. penetrans* and may be used to follow the evolution of the real pool of bacteria either in native suppressive soils or in inoculated ones.

Keywords: Immunological detection; Soil; Unculturable bacterium; *Pasteuria penetrans*

1. Introduction

Pasteuria species are obligate parasites of several plant parasitic nematodes [1] and daphnia [2]. They are Gram-positive and belong to an endospore-forming bacteria group but their taxonomy remains unclear. Three nematode parasitic species have been characterized according to their morphological diversity (size and shape) and host specificity: (i) *Pasteuria thornei*, parasite of root lesion nematodes *Pratylenchus* spp. [3]; (ii) *Pasteuria nishizawae*, parasite of cyst nematodes *Heterodera* spp. and *Globodera* spp. [4]; (iii) *Pasteuria penetrans*, parasite of root-knot nematodes *Meloidogyne* spp. [5]. More recently, new isolates parasitizing *Heterodera goettingiana* [6] and *Belonolaimus longicaudatus* [7] were identified as putative new species. Recent phylogenetic studies based on rDNA sequences placed the *Pasteuria* group closed to *Bacillus* [8,9].

The bacterium has potential to act as a biological control agent either by restraining the nematode migration toward the roots or by reducing their reproduction [10,11]. *P. penetrans* was the most studied organism in producing *Meloidogyne* suppressive soils [12,13]. This nematode is the most economically important nematode pest in the world, especially in tropical and subtropical countries [14,15].

The parasitism of *Meloidogyne* species by *P. penetrans* has two main steps. First, an external adhesion of free spores of *P. penetrans* on the nematode cuticle occurs in the soil and depends on attachment compatibility between bacterial spores and nematodes [16] and on the population densities of each organism [12]. Second, an internal infection occurs when nematodes have reached the roots and began to feed in: spores germinate, penetrate through the nematode cuticle, colonize the nematode coelome [5–17] and form new spores which will be released in soil after nematode death.

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In order to develop integrated pest management of *Meloidogyne* spp. with *P. penetrans*, it is of fundamental importance to understand the nature of host specificity and heterogeneity of spores which depend on individual attachment mechanisms [16,18–20], which in turn affects the epidemiology of the bacterium in the soil. As the culture of *P. penetrans* has been unsuccessful [21], it can not be detected and enumerated in soils by standard techniques as most probable number enumeration (MPN) and plating techniques [22]. Usually, quantification of *P. penetrans* in soils relies on the numeration of infested juveniles of *Meloidogyne* spp. and of spores attached to their cuticle [23,24].

The use of 16S rDNA sequences from *Pasteuria* spores to enumerate their density in soil is difficult because spore permeabilization and lysis directly in soils limits considerably the use of molecular tools to enumerate spores from soil samples. Therefore, alternative techniques are required. Immunofluorescence microscopy and ELISA are widely used in food industry for quality control purposes including pathogen and toxin detection [25]. These immunological methods have also been used with success in different microbial environments such as soils, sediments, plants, root systems, waste water, sea water, etc. [26–29] and provided results as sensitive as molecular ones [30–33].

The aim of this work was to quantify *P. penetrans* spores in soil samples, based on immunological techniques. Extraction of spores that adjusts their quantification was first examined, and then two immunological methods (immunofluorescence and ELISA) were studied. Analyzing these techniques in different soil conditions, technical advantages and difficulties were discussed in order to provide accurate applications.

2. Materials and methods

2.1. Pasteuria endospores and bacterial strains

Four weeks old tomato (*Lycopersicon esculentum* cv. Roma) seedlings were transplanted in 1 dm^3 pots previously filled with a soil sampled in an African egg-plant (*Solanum aethiopicum* cv. Soxna) cropped field in Sénégal (experimental station of the Ecole Nationale Supérieure d'Agriculture, Thiès), naturally infested with *Meloidogyne javanica* juveniles parasitized by *P. penetrans* (isolate ORS-21414-Sen). Five weeks after transplantation, the plants were uprooted and washed with tap water. Females of *M. javanica* infected with spores of *P. penetrans* were dissected from the root galls and crushed in distilled water in 1.5 ml Eppendorf tubes. The suspensions were sieved through a 10 μm sieve in order to remove nematode debris and 5×10^6 spore ml^{-1} stock suspensions were stored at −20°C as inoculum. Other *P. penetrans* isolates sampled in Sénégal and in other countries were obtained as described above (except the isolates Pp1 and 'Sayre' which were provided as dry root powders and mixed to soil) and various bacterial strains cultured in laboratories were also used (Tables 1 and 2).

2.2. Inoculation in different soils

Three soils were sampled in vegetable cropped fields in Sénégal. A sandy soil (1.1% clay, 2.2% silt, 96.7% sand, and 2.4‰ organic carbon) was sampled from the experimental station of the Institut Sénégalais de Recherche Agricole (Cambérène). A clay soil (57.1% clay, 22.4% silt, 21.9% sand, and 26.8‰ organic carbon) was sampled in the west valley of the Sénégal river (Podor). The third soil, characterized by a sandy–clay texture (10.3% clay, 4.1% silt, 85.6% sand, and 2.9‰ organic carbon), was sampled in the same field as described above.

The experiments were based on 1 ml suspensions of 5×10^4 *P. penetrans* ORS-21414-Sen spores inoculated into 1 g of soil that had been autoclaved (2 h, 120°C) in sterile vials. In order to study the effect of the soil texture on the spore detection, 1 ml suspensions with 5×10^4 spores or with 15×10^5 spores were inoculated to 1 g or to 30 g of soil respectively. In order to correlate the number of spores recovered with the number of spores inoculated, serial inocula from 0 to 10^6 spores per g of soil were inoculated. Each soil–inoculation combination was replicated four times.

2.3. Extraction of the spores

The spores were extracted from the inoculated soils according to three different methods.

2.3.1. Gentle shaking/sieving extraction

1 g of soil was manually dispersed during 1 min in 4 ml of distilled water. Then, the soil suspension was sieved (20 and 10 μm) and washed with distilled water. The soil filtrate was kept at 4°C.

2.3.2. Aqueous two-phase partitioning (A2PP) extraction [34]

1 g of soil was shaken (100 rpm for 10 min) in 4 ml of a 0.1% sodium cholate with 2.4 g of Amberlite® resin (Sigma, France) solution. The soil suspension was then sieved through a 20 μm sieve and the filtrate was mixed with 10 g of Dextran® (Sigma, France) 20% (w/w) and 15 g of polyethylene glycol (PEG) 8000 40% (w/w). Partitioning was performed at 4°C for 2 h. The upper PEG phase containing the spores of *P. penetrans* was diluted with distilled water (4:5, v/v) and centrifuged ($10\,000\times g$ for 30 min at 20°C). The pellet was washed twice in distilled water and kept at 4°C.

2.3.3. Differential centrifugation extraction with PEG [35]

1 g of soil was shaken (100 rpm for 2 h) in 4 ml of 0.1%

sodium cholate and 2.5% (w/v) PEG 6000 with 0.2 g of Chelex® 100 resin (Sigma, France). The soil suspension was centrifuged ($177\times g$ for 30 s) in order to remove the coarsest soil particles. The supernatant containing the spores of *P. penetrans* was sieved through a 20 μm sieve to remove both resin and residual soil particles. The filtrate was centrifuged ($3500\times g$ for 15 min) and the pellet was resuspended in 10 ml of distilled water and kept at 4°C.

Each experimental condition was replicated four times.

2.4. Polyclonal antisera

For immunization, the inoculum spore suspensions were cleaned by loading about 10^8 spores on a four-step sucrose gradient (2 ml of 87, 82, 75, and 67% w/v), and spun ($25\,000\times g$, 2 h, 4°C). They were then washed three times and stored in sterile PBS (10 mM sodium phosphate buffer, pH 7.4; 0.9% sodium chloride). To ascertain the absence of contaminating bacteria, purified spore suspensions and their dilutions were plated on Luria Broth medium and examined microscopically.

Pre-immune serum was collected on rabbits maintained in IACR (Harpenden, UK). They were immunized with a suspension of purified spores (5×10^6 spores in 500 μl PBS) of *P. penetrans* ORS-21414-Sen injected in the peritoneum. A boost injection was performed with the same spore suspension into the rabbits 1 month after the previous one. Two months later, blood samples were collected. Another polyclonal antiserum (anti-PP1) was raised by the same procedure against spores of *P. penetrans* population PP1 [18]. Pre-immune and immune (anti-ORS-21414-Sen and anti-PP1) sera aliquots were stored at −20°C.

2.5. Immunofluorescent quantification of the spores

3 μl of each of the spore suspensions (inoculum suspensions or soil extracts) were coated on multiwell slides (ICN Biomedical Inc., USA) and washed with PBS. Then, 10 μl of either pre-immune or anti-ORS-21414-Sen or anti-PP1 sera diluted in PBS (1:1000, v/v) supplemented with 0.1% (v/v) Tween and 5% (w/v) milk powder (PBSTM) were added. After a 2 h incubation at 37°C, the smears were washed three times with PBS. Then, 10 μl of anti-rabbit IgG (Sigma, France) conjugated with fluorescein isothiocyanate (FITC) in PBSTM (1:50, v/v) were added. After another 2 h incubation at 37°C, the smears were washed three times in PBS and mounted in Citifluor® (Agar Scientific, UK). Four replicated counts of the spores were performed using an epifluorescence microscope (UV 455 nm, magnification-$\times$2000).

In order to verify its specificity, the anti-ORS-21414 polyclonal serum was applied, according to the same process, on various *Pasteuria* isolates (Table 1), on bacterial strains (Table 2) and on non-identified colonies isolated from the native soil by dilution plating on tryptic soy broth medium. Cross reactivity was determined on four replicates of 100 bacteria from each spore isolates, bacterial strains or non-identified colonies, examined microscopically for their immunofluorescence with the anti-ORS-21414 polyclonal serum. Non-identified colonies were isolated from the native soil. In order to compare the efficiency of the extraction methods used, the spore recoveries were estimated on the sandy–clay soil by detecting them with the polyclonal anti-PP1 serum. The effect of the different soil textures on spore recovery was studied by using the gentle shaking/sieving extraction method, and by detecting them with the anti-PP1 polyclonal serum. Finally, the anti-ORS-21414-Sen and anti-PP1 polyclonal sera were applied on extracts of four sandy–clay soil units randomly sampled from the African egg-plant cropped field (see above), in order to compare their efficiency and to evaluate the native spore concentration range in this soil. In all experiments, native populations of spores were observed in non-inoculated soils and taken in account to evaluate the real recovery of the spores inoculated.

2.6. ELISA quantification of the spores

The antigen coating plate ELISA technique (ACP ELISA), which was efficient for the quantification of *Bacillus polymyxa* spores in soil samples [36,37], was used. NUNC™ Maxisorp F96 microtiter plates were coated with 50 μl of either inoculum spore suspensions or spore soil extracts (gentle shaking/sieving method only) overnight at room temperature. The plates were then washed three times with PBS supplemented with 0.1% (v/v) Tween (PBST), and 50 μl of either anti-ORS-21414-Sen or anti-PP1 sera diluted in PBSTM (1:1000, v/v) were added. After a 1.5 h incubation at room temperature, the plates were washed three times in PBST again, and 50 μl of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, France) were added (1:1000, v/v). After a 2 h incubation, the plates were washed three times with PBST. The alkaline phosphatase activity was revealed with 200 μl of 1.0 mg ml^{-1} paranitrophenyl phosphate (pNPP Sigma Fast tablets, France) in the dark, and read at 405 nm with a Multiskan® plate reader (Labsystems, France) after a 20 min incubation. In order to detect natural phosphatase activities in the soil, pNPP was applied to native soil extracts. All ELISAs were repeated three times.

2.7. Statistical analysis

Data were analyzed according to the Kruskall and Wallis test and compared with ANOVA analysis. Percentages were transformed by Arcsin (sqtr) before analysis.

Table 1
P. penetrans isolates used for immunofluorescence cross reactions with the anti-ORS-21414-Sen polyclonal serum

Isolates	Geographical origin	*Meloidogyne* host of origin	Host plant of origin
ORS-21414-Sen	Sénégal	*M. javanica*	*L. esculentum*
ORS-48904-Sen	Sénégal	*M. mayaguensis*	*L. esculentum*
ORS-48905-Sen	Sénégal	*M. mayaguensis*	*Solanum melongena*
ORS-53602-Sen	Sénégal	*M. incognita*	*Musa acuminata*
ORS-11570-WI	Martinique	*M. incognita*	*S. melongena*
ORS-KM23-Ind	Indonesia	*M. incognita*	unknown
ORS-NC1-Ncal	New Caledonia	*M. javanica*	*L. esculentum*
ORS-Pp24	unknown	*Meloidogyne* sp.	unknown
Pp1	Australia	*M. incognita*	*L. esculentum*
'Sayre'	Australia	*M. arenaria*	*L. esculentum*

3. Results

3.1. Immunofluorescent detection of the spores of P. penetrans in the inoculated soil samples

3.1.1. Polyclonal specificity and cross reactions

P. penetrans spores were easily detected in the different soil extracts with either the anti-ORS-21414-Sen polyclonal serum or the anti-PP1 one, showing a hard green fluorescence under UV light (Fig. 1). No cross reaction either with the microflora isolated from the soils or with the bacterial strains tested was observed with the anti-ORS-21414-Sen polyclonal serum. However cross reactions were observed with all the *P. penetrans* isolates used (Table 3), the spores from Sénégal being more fluorescent than the exotic spores, except for the strain ORS-11570-WI.

3.1.2. Extraction techniques

A significant effect of the extraction method was recorded on the percentage of spores recovered from the inoculated sandy–clay soil and detected with the anti-PP1 polyclonal serum (Fig. 2). For the gentle shaking/sieving method, extracted spores amounted to 94% of the inoculum, while recoveries with A2PP and PEG extraction methods amounted to 34.5 and 61.9% respectively.

3.1.3. Detection in different media

The anti-PP1 polyclonal serum detected all the spores in the inoculum suspensions. It detected about 5200 spores per g of soil in the non-inoculated sandy–clay soil sample naturally infested with *P. penetrans*. Comparing the detection of the spores in inoculated soils, less than 15% of the spores inoculated to 30 g of clay soil was recovered (Fig. 3). For each inoculum condition, the recovery of the spores did not show any difference between the sandy soil and the sandy–clay soil. But, 37.4 and 39.6% more spores were detected in the sandy–clay soil and in the sandy soil respectively when 5×10^4 spores were inoculated in 1 g of soil than when 15×10^5 spores were inoculated in 30 g of soil.

3.2. Immunofluorescent detection of the spores of P. penetrans in the field soil samples

Irrespective of the field sandy–clay soil samples processed by the gentle shaking/sieving extraction method, spore detection by both polyclonal anti-ORS-21414-Sen and anti-PP1 sera did not show any difference (Fig. 4). The spore densities detected in the soil varied significantly from 5000 to 145 000 spores per g of soil between samples.

3.3. Correlation between inocula and recovery levels of spores from soils

In the soils inoculated and processed by the gentle shaking/sieving extraction method, the detection with the anti-ORS-21414-Sen polyclonal serum revealed that the lowest inocula (10^3 spores per g of soil) were slightly overestimated and the inocula $\leq 10^5$ spores per g of soil were slightly underestimated, more in the sandy–clay soil than in the sandy soil (Fig. 5).

Table 2
Bacterial strains used for immunofluorescence cross reactions with the anti-ORS-21414-Sen polyclonal serum

Bacterial species	Strain	Collection
Aeromonas caeloceticus	138	IACR, UK
Aeromonas sp.	155	IACR, UK
Aeromonas sp.	B1	IACR, UK
Aeromonas sp.	B2	IACR, UK
Aeromonas sp.	B3	IACR, UK
Bacillus anthracis		CEB, France
Bacillus cereus		CEB, France
Bacillus mycoides		CEB, France
Moxarella osloensis	48	IACR, UK
Providencia rettgeri	77	IACR, UK
Pseudomonas fluorescens	140	IACR, UK
P. fluorescens	A1	IACR, UK
P. fluorescens	A2	IACR, UK
Rhizobium sp.	ORS-1043	IRD, Sénégal
Rhizobium sp.	ORS-1073	IRD, Sénégal

CEB, Centre d'Etudes du Bouchet; IACR, Institute of Arable Crop Research; IRD, Institut de Recherche pour le Développement.

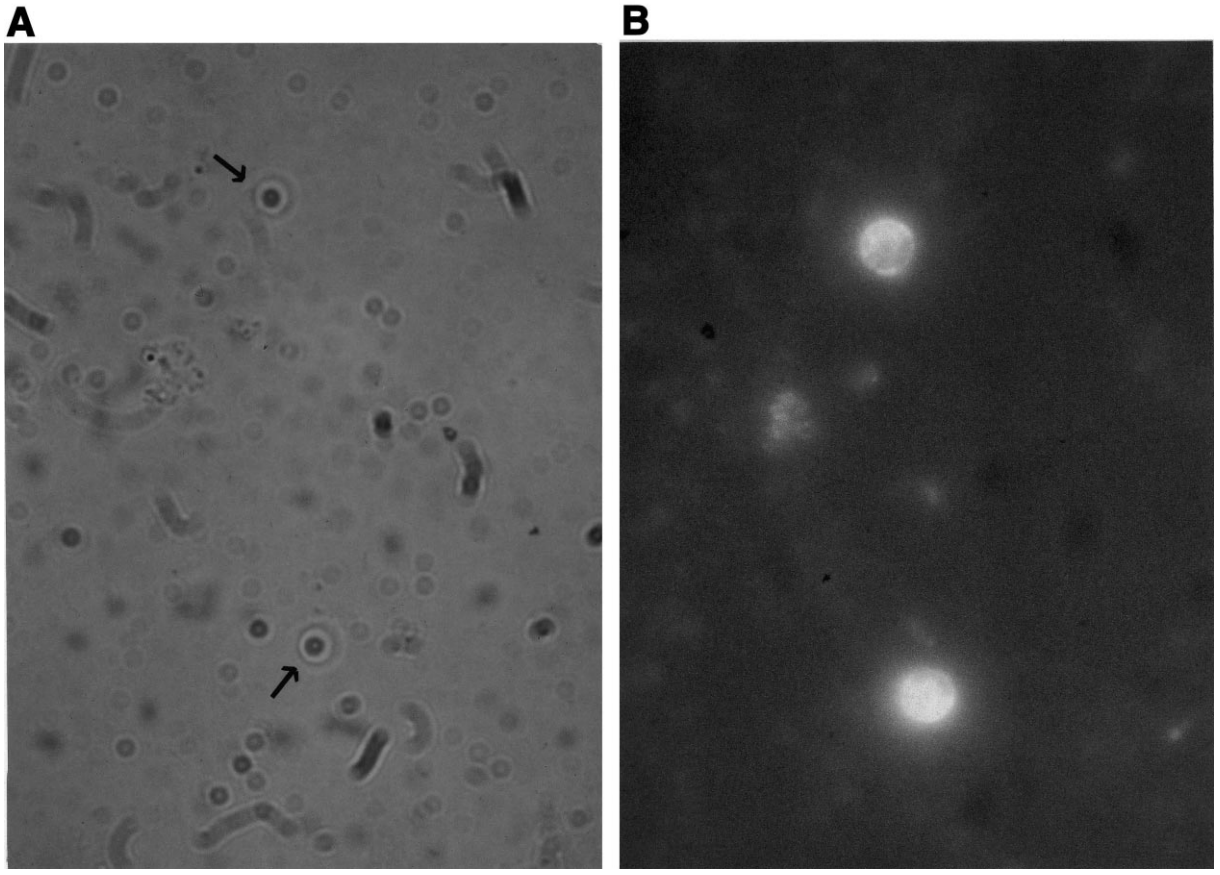

Fig. 1. Spores of *P. penetrans* ORS-21414-Sen (indicated by arrows) observed under light (A) and fluorescence (B) microscopy in soil extracts and detected with the anti-ORS-21414-Sen polyclonal serum (1 cm = 5.7 μm).

3.4. ELISA quantification of the spores of P. penetrans

No native alkaline phosphatase activity was detected in the soil extracts. The minimal detection thresholds in the soil extracts were between 250 and 1000 spores per ml (i.e. 5×10^3 to 2×10^4 spores per g of soil). The ELISA curves showed a significant correlation between the phosphatase activity and the spore concentration, either in the inoculum suspension or in the soil extracts, ranging from 5×10^3 to 5×10^5 spores per ml (i.e. 10^5 to 10^7 spores per g of soil) (Fig. 6). Between these low and high concentration thresholds, the ELISA quantification was as efficient in the inoculum suspension as in the sandy and the sandy–clay soil extracts.

Table 3
Cross reactions of the anti-ORS-21414-Sen polyclonal serum against the *P. penetrans* isolates (four replicates per spore isolate)

Isolates	Fluorescence (% of 100 spores observed)		
	High	Low	No
ORS-21414-Sen	36	64	0
ORS-48904-Sen	36	58	6
ORS-48905-Sen	32	62	6
ORS-53602-Sen	12	60	8
ORS-11570-WI	52	48	0
ORS-KM23-Ind	0	92	8
ORS-NC1-Ncal	14	86	0
ORS-Pp24	4	76	20
Sayre	8	92	0

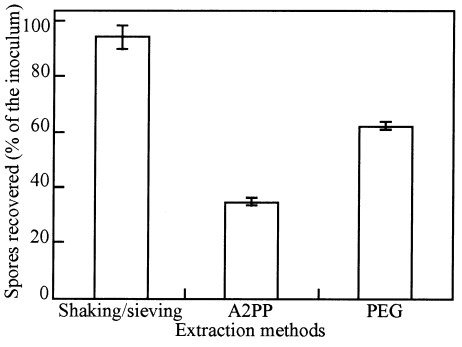

Fig. 2. Percentages of spores of *P. penetrans* ORS-21414-Sen recovered from the inoculated sandy–clay soil dispersed with three different methods and detected by immunofluorescence microscopy with the anti-PP1 polyclonal serum (four replicates for each condition; bars represent standard errors, $P = 0.05$).

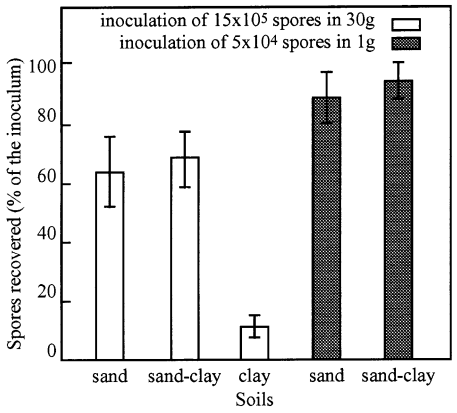

Fig. 3. Percentages of spores of *P. penetrans* ORS-21414-Sen recovered from different soils dispersed with the gentle shaking/sieving extraction method, and detected with the anti-PP1 polyclonal serum by immunofluorescence microscopy (four replicates for each condition; bars represent standard errors, $P = 0.05$).

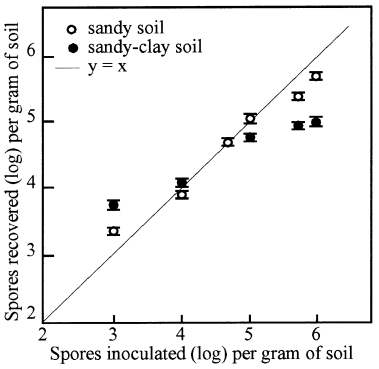

Fig. 5. Relationship between the number of spores of *P. penetrans* inoculated in soils and the number of spores extracted with the gentle shaking/sieving method and detected with the anti-ORS-21414-Sen polyclonal serum by immunofluorescence microscopy (four replicates for each condition; bars represent standard errors, $P = 0.05$).

4. Discussion

4.1. Detection of P. penetrans spores in soil

As *P. penetrans* is an unculturable bacterium, its detection compels to develop phenotypic or molecular tools. Considering the emerging knowledge on the *P. penetrans* genome (only three 16S sequences available on GenBank database) and the difficulties to extract DNA from spores without contaminating it with other microorganism nucleotides [8,9], detection was performed using phenotypic characteristics of spores.

Reactions of the anti-ORS-21414-Sen polyclonal serum against various bacterial strains and different *P. penetrans* isolates sampled in vegetable fields in Sénégal and in other countries, indicated that this serum is specific to *P. penetrans*, but not only to the isolate ORS-21414-Sen. As it was previously demonstrated [19,38,39], some *Pasteuria* spore surface antigens would be common to various isolates. So, the anti-ORS-21414-Sen and anti-Pp1 polyclonal sera would share the same antibodies as they are able to detect ORS-21414-Sen spores in the soil. Therefore, these anti-*P. penetrans* polyclonal sera could be used to detect and quantify *P. penetrans* isolates, but their specificity would be limited to *P. penetrans sensu stricto* parasitizing *Meloidogyne* populations. Indeed, it was previously observed that the anti-ORS-21414-Sen polyclonal serum was enable to detect *Pasteuria* populations isolated from other Tylenchid nematodes sampled in sahelian fallows and forests [40]. However, the use of monoclonal antibodies which are specific to one antigen could probably allow the identification at the population or sub-population level possible [38].

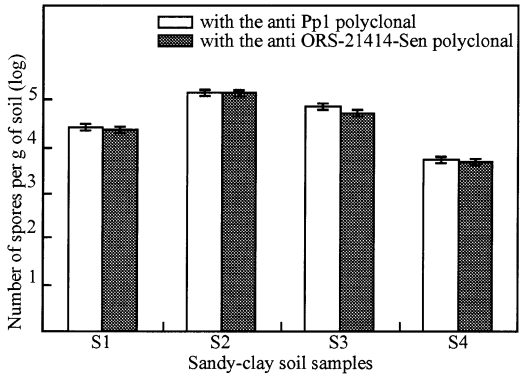

Fig. 4. Number of spores of *P. penetrans* ORS-21414-Sen recovered from four samples of the field sandy–clay soil dispersed with the gentle shaking/sieving extraction method, and detected with the two polyclonal sera by immunofluorescence microscopy (four replicates for each condition; bars represent standard errors, $P = 0.05$).

4.2. Spore extraction from soil

Extraction of microorganisms from soil is a key step for their quantification, and soil dispersion in aqueous suspensions is the obligate way to release them from soil particles. Dispersion methods differ according to the disrup-

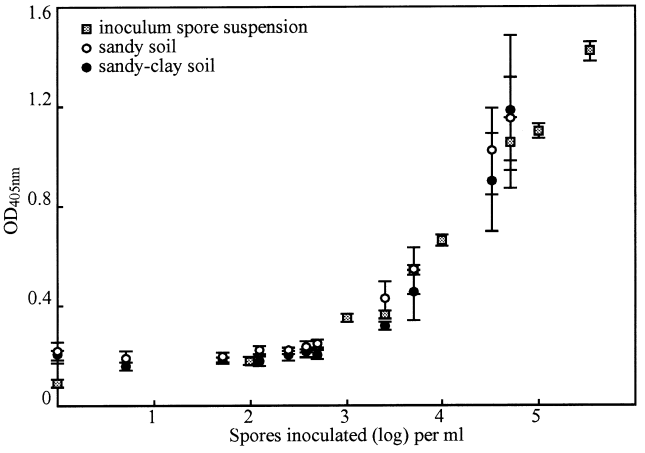

Fig. 6. ELISA quantification with the anti-ORS-21414-Sen polyclonal serum and alkaline phosphatase of spores of *P. penetrans* in diluted spore suspensions or soils dispersed with the gentle shaking/sieving extraction method (three replicates for each condition; bars represent standard errors, $P = 0.05$).

tive agent used and to the dispersion strength and time assigned. Strong physical (as sonication or shaking with marbles) and chemical (as with sodium hydroxide) dispersions lead to release most of the microorganisms inhabiting soils by destroying the smallest aggregates, and, consequently, by releasing the bacteria that live inside or strongly adhere to them [41]. In this study, the gentle shaking/sieving extraction gave a higher spore recovery than A2PP and PEG. Even spore recoveries with A2PP and PEG are close to those obtained with other bacteria [34,35], these techniques need more technical steps and manipulations than the gentle extraction and then provide more opportunity for loss of spores. On the other hand, because of their negative charge [42,43], the spores could adhere to the colloidal particles [44] and be released by the exchange resins, being then carried out by the useful supernatants. These hydrophobic and electrochemical phenomena were confirmed by the effect of $CaCl_2$ (used to separate bacteria from soil particles by flocculation [37,45]), which did not improve spore recovery (data not shown). They also could explain that less spores were recovered from the clay soil than from the sandy soils. Gentle shaking/sieving and PEG methods, which are known to eliminate more coarse soil particles, should be more effective for sandy soils than A2PP. However, A2PP provided samples with less contaminating soil debris with the sandy–clay soil than the other extraction methods, and therefore may be used for qualitative rather than for quantitative detection. Therefore, the more energetic dispersion techniques should be applied to heavy soils, but care should be taken that these techniques should not affect the integrity of the spores, especially their exosporium and surface coat where the antigens are located [18], and thereby could affect immunological detection by the polyclonal sera.

In light soils, the gentle shaking/sieving method is sufficient to quantify the spores available for attachment on nematodes, as quite all of them are free in the soil porosity or very slightly adsorbed on the pore walls. In more compacted soils, spores, which are not released with a weak dispersion, are likely to be trapped in the soil microporosity or in aggregates, or strongly adsorbed on particles [46], and therefore unlikely to play an active role on the nematode parasitism because *Meloidogyne* juveniles migrate in size pores >10 μm, and ionic or hydrophobic competitions should act on the detachment of spores from the soil matrix [47].

4.3. Sensitivity of the quantification methods

Detection threshold and sensitivity of the quantification methods depend mainly on the soil matrix and the efficiency of the extraction procedure used. Moreover, it is difficult to compare exactly detection threshold between several studies because units are seldom uniform (i.e. CFU ml^{-1}, bacteria ml^{-1}, bacteria g^{-1}) and sample sizes are different. In most of the studies, quantification was designed to monitor cultivated or genetically engineered microorganisms released in the environment [48]. In contrast, only a few studies have developed such quantification techniques on unculturable bacteria like *P. penetrans* [31]. In this study, ELISA thresholds obtained in spore suspensions and soil extracts were slightly better than those obtained (1000 spores ml^{-1} vs. 1600 spores ml^{-1}) on *P. nishizawae* spore suspensions from *Heterodera* cysts [49]. Moreover, the ELISA results presented here obtained levels of detection that were more sensitive than those obtained on *B. polymyxa* spores (i.e. 6×10^4 spores ml^{-1}) [37].

The immunofluorescent detection of *P. penetrans* spores in the vegetable field where the ORS-21414-Sen isolate was sampled, revealed a significant variation of the spore density ranging from 5000 to 145 000 spores per g. As ELISA could also detect such spore ranges, it would be interesting to use it for field distribution and ecological studies in order to quantify the suppressive power of the *P. penetrans* populations [23] and their fluctuations according to their dependence with nematode population densities [50] and soil environment [47].

Immunological methods developed here, especially ELISA, were sensitive, rapid and useful for *P. penetrans* enumeration in soil samples. By applying these methods to plot and field studies, epidemiological knowledge on these unculturable bacteria could be significantly increased, and therefore, population dynamics of *P. penetrans* could be easily monitored to improve nematode biocontrol management.

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