Original article

Avirulence of Viable But Non-Culturable *Listeria monocytogenes* cells demonstrated by in vitro and in vivo models

Jean Michel CAPPELIER[a]*, Valérie BESNARD[a], Sylvie ROCHE[b], Nathalie GARREC[a], Etienne ZUNDEL[b], Philippe VELGE[b], Michel FEDERIGHI[a]

[a] Unité Mixte de Recherche SECALIM INRA-ENVN, École Nationale Vétérinaire de Nantes, BP 40706, Route de Gachet, 44307 Nantes, France
[b] Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France

Abstract – The virulence of Viable But Non-Culturable (VBNC) cells of 4 strains of *Listeria monocytogenes* was investigated in both a human adenocarcinoma cell line (HT-29) and a mouse model. LO 28, ATCC 19115 and CNL 895807 strains of *Listeria monocytogenes* became VBNC when incubated in microcosm water at 20 °C and Scott A strain at 4 °C. No culturable bacteria were detected in the VBNC state, although 10^4 active cells/mL were found by the Direct Viable Count (DVC) and CTC-DAPI double staining methods. A comparison of virulence in both human adenocarcinoma cell line HT-29 and the mouse model showed that culturable controls were more virulent than VBNC cells, which appeared to be avirulent regardless of the virulence methods applied. Pathogenicity was tested in each model and was lost concomitantly with culturability, whereas some cells were still metabolically active (determined by CTC and DVC). Moreover, amplification of a 388 bp fragment with Immunocapture-PCR revealed the presence of *Listeria monocytogenes* DNA in all mixed spleen samples after intravenous injection of VBNC cells. These results demonstrate that VBNC cells were present in the mouse spleens. The results of the study suggest that *Listeria monocytogenes* strains might remain in the aquatic environment for prolonged periods in the VBNC state but these cells were not pathogenic in the conditions tested. These findings demonstrate the value of VBNC studies and show the need to investigate the role of VBNC cells in environmental transmission of *Listeria monocytogenes*. Further studies are needed in order to investigate the virulence of VBNC cells of *Listeria monocytogenes* after recovery of a culturable state.

VBNC / virulence / *Listeria monocytogenes* / plaque forming assay

1. INTRODUCTION

Listeria monocytogenes is a food-borne pathogen that has become a significant public health concern. The causative agent of listeriosis is ubiquitous in the environment. It has been recognized as an animal pathogen since the 1920s, and in the past two decades it has been implicated in numerous outbreaks of food-borne illness in humans [1, 32]. Some sections of the population are predisposed to development of listeriosis due to the presence of chronic illness, suppression of the immune system, pregnancy,

---

* Corresponding author: cappelier@vet-nantes.fr

or extreme youth or old age (under 1 year or over 60 years) [19]. This gram-positive, psychrophilic bacterium can cause meningitis, abortion and perinatal septicemia in humans. Listeriosis is fatal in up to 30% of the cases [14, 16].

In response to different stress factors, *Listeria monocytogenes* is known to enter into a Viable But Non Culturable (VBNC) state [4]. Bacteria in the VBNC state cannot be recovered when standard bacteriological media and procedures are employed, but they may regain culturability under appropriate conditions [21, 30, 37]. Until recently, the traditional plate culture method was used almost exclusively to estimate the number of viable bacterial cells in a given sample. However traditional plating methods may underestimate the number of viable bacteria because of the VBNC state, as demonstrated in a variety of bacterial species by newer methods involving direct microscopy examination of samples that indicate active respiration. This distinction between viability and culturability is especially critical for pathogens, because loss of culturability may not guarantee loss of pathogenicity.

There is thus uncertainty regarding the health risk posed by VBNC cells [29]. Some authors have demonstrated pathogenic effects caused by VBNC cells [13], whereas others have claimed the concomitant loss of culturability and pathogenicity [20].

In this study, *Listeria monocytogenes* strains exposed to nutrient starvation were investigated for colony forming ability, changes in cellular state (viability staining techniques) and virulence in both a human adenocarcinoma cell line (HT-29) and a mouse model.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Four *Listeria monocytogenes* strains (Scott A, CNL 895807, ATCC 19115, LO 28)

were provided by the laboratory of Infectious Pathology and Immunology, INRA Nouzilly, France. For each test, the strains were grown for 24 h at 37 °C on Plate Count Agar (PCA) (Biokar, Beauvais, France). Two subcultures were performed under similar conditions and the cells were placed in microcosm water.

2.2. Experimental model to obtain VBNC cells of *Listeria monocytogenes*

The microcosm water described by Rollins and Colwell [28] was used to induce VBNC *Listeria monocytogenes* cells. The predominant factors of VBNC state induction have been previously determined [6]. After two 24 h subcultures in PCA at 37 °C, the cells were collected, washed and suspended in bottles containing 500 mL of filtered sterilized distilled water (Nalgene 0.2 μm, Bioblock, Strasbourg, France) adjusted to pH 5.0 ± 0.1 (HCl 0.1 mol/L) to obtain a final concentration of about 10^6 bacteria/mL.

The bottles were then left in darkness for 10 weeks at 20 °C for strains CNL 895807, ATCC 19115 and LO 28 and at 4 °C for strain Scott A, with gentle shaking at 100 rpm. All experiments were performed in triplicate.

2.3. Culturability and cellular state tests

2.3.1. Spread plate counts and culturability assays

Culturability was assayed by spreading on plate counts. Serially diluted samples (0.1 mL) in peptone water were spread in triplicate on PCA. After 48 h of incubation at 37 °C, the number of colony-forming units (cfu) at the appropriate dilution were counted. When culturable cells were less than 300 per mL, 0.1 mL samples of microcosm water were spread on 10 Petri-dishes containing PCA.

The state of VBNC cells was checked in different media (BHI, peptone water, DMEM, FCS). When no colony was observed in 1 mL but metabolic activity was detected by DVC or CTC DAPI, 10 mL of microcosm were centrifuged for 15 min at 10 000 g. After centrifugation, the pellets were placed in 10 mL Brain Heart Infusion (BHI) (Biokar, Beauvais, France), 9 mL peptone water (MERK, Darmstadt, Germany), 10 mL Dulbeccos Modified Eagle Medium 4.5 g/L glucose (DMEM; Life Technologies, Cergy-Pontoise, France) and 10 mL DMEM supplemented with 10% (v/v) fetal calf serum (FCS, Life Technologies). The solutions were maintained at 37 °C. After 24, 48 and 72 h, 0.1 mL aliquots were spread on PCA. The petri-dishes were placed at 37 °C for 72 h. The absence of colonies on PCA after incubation meant that bacterial cells present in the microcosm water were considered to be Non-Culturable.

2.3.2. Characterization of metabolic activity

– Direct Viable Count (DVC)

A modified technique of Kogure et al. [17] was used [5] to enumerate VBNC cells of *Listeria monocytogenes*.

Because of presence of ciprofloxacin, metabolically active cells absorb nutrients and begin to elongate without dividing. The size of metabolically active cells therefore increases, while the size of non-active cells remains unchanged. These differences in cell size are visible on epifluorescence microscopy after staining. Briefly, 1 mL of microcosm water was added to 9 mL of BHI, 100 μL yeast extract at 25 mg/L (Difco, Detroit, Michigan, USA) and 5 μL ciprofloxacin solution (Bayer, Puteaux, France) at 2 mg/mL. After 7 h of incubation at 37 °C, the mixture was filtered through a 0.2 μm pore size polycarbonate filter (Bioblock, Strasbourg, France) and stained for 5 min with a DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) solution (Inter-

chim, Montluçon, France) at 5 μg/mL. Finally, the filter was air-dried and mounted in non-fluorescent immersion oil (Nikon, Paris, France), and a coverslip was added before observation with an epifluorescence microscope (Olympus BX40, Rungis, France), equipped with a BW2-RFL-T3 100-W light source. Counting was performed randomly on the basis of 10 microscope fields per filter.

Four filters were counted for each sample. Only cells elongated to at least twice the cell length in fresh culture were counted as viable cells.

Total enumeration was performed by filtering 1 mL of microcosm water through a 0.2 μm pore size polycarbonate filter and staining with DAPI for 5 min. The experiments were conducted in triplicate.

– CTC-DAPI double staining

A modified technique of Rodriguez et al. [27] was used to detect cellular activity in bacteria. Counterstaining CTC (5-cyano-2;3-ditolyl tetrazolium chloride; Polysciences Inc., Warrington, Pennsylvania, USA) with DAPI allowed simultaneous determination of total (red and blue fluorescent) bacteria and viable bacteria. All the bacteria of the sample are stained blue by DAPI, and only active bacteria that are able to reduce CTC show intracellular red fluorescent precipitate formazan crystals. One milliliter of microcosm water was centrifuged for 15 min at 10 000 g. After centrifugation, the pellets were recuperated in 0.5 mL of distilled filtered water (pH 6.0), with 100 μL at 0.05 g/mL solution of pyruvic acid (Sigma, Saint-Quentin-Fallavier, France) and 0.5 mL of BHI and 100 μL of CTC at 15 mg/mL.

After 3 h of incubation at 37 °C, the mixture was filtered through a 0.2 μm pore size polycarbonate black membrane filter (Bioblock, Strasbourg, France) and stained for 5 min with a DAPI solution at 5 μg/mL. Microscopy observation was performed as above. Three filters were counted for each sample. The results are expressed as the number of corresponding bacteria per mL of the original

sample. The experiments were performed in triplicate.

2.4. Virulence tests

The *Listeria monocytogenes* strains (culturable and VBNC cells) were tested for their invasive character with HT-29 cell monolayers. Plaque-forming and entry ability of VBNC cells were analyzed and compared to culturable cells. Resuscitation of VBNC cells was also assessed after contact between VBNC cells and HT-29 cells. The strains were also tested for their potential to colonize mouse spleens (Swiss female OF1).

2.4.1. In vitro virulence assays

– Cells

The human adenocarcinoma cell line HT-29 (ECACC N° 85061109, Salisbury, United Kingdom) was used for in vitro virulence assays. The cells were grown in 75 cm^2 plastic tissue culture flasks (Nunc, Life Technologies) in culture medium: DMEM supplemented with 10% FCS (v/v) and 2 mM L-glutamine (Life Technologies). Antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin; Sigma, France) were routinely added to the culture medium except for the virulence assays. The cells were maintained in a humidified incubator (Heraeus, Les Ulis, France) at 37 °C under 5% CO_2.

– Plaque-forming assay

The ability of the strains to form plaques was tested using the technique described by Van Langendonck et al. with some modifications [35]. Trypsinized HT-29 cells (9.10^5) were deposited in 6-well tissue culture plates (Falcon, AES Laboratoire, Combourg, France) and cultured in culture medium to obtain a constant proliferation rate [36]. The cells were incubated for 4 days to obtain almost confluent monolayers and then in antibiotic-free medium for 24 h. For positive controls, culturable *Listeria* strain cells were grown overnight on BHIA (Brain Heart Infusion Agar, Difco, Becton Dickinson, Meylan, France) slopes and bacterial concentrations were standardized turbidimetrically and diluted appropriately in DMEM in order to obtain a bacterial concentration of 10^4 cfu/mL. For VBNC cells, 1 mL of microcosm water containing 10^4 metabolically active cells per mL was centrifuged for 5 min at 10 000 *g*. The Pellets were suspended in 1 mL of DMEM after centrifugation.

HT-29 cells were infected with 10^4 culturable bacteria/mL (positive controls) and 10^4 VBNC cells/mL and incubated for 2 h at 37 °C in a humidified incubator, then incubated for 1 h 30 min in the presence of 100 μg/mL gentamicin (Sigma, St. Louis, USA) in culture medium. Each well was emptied and then overlaid with an agarose gel containing 0.48% indubiose (Serva, BioWhittaker, Fontenay-sous-Bois, France) in culture medium supplemented with 10 μg/mL gentamicin. The same medium was then added to prevent HT-29 cell starvation. After 72 h at 37 °C the number of plaques was assessed. Enumeration of deposited culturable *Listeria* strains was performed on TSA plates (Tryptone Soy Agar, Bio-Mérieux, Marcy-L'Étoile, France). Enumeration of deposited VBNC *Listeria* strains was performed by microscopy observation.

The results were expressed as the number of plaques obtained for 10^4 *Listeria* deposited per well.

– VBNC cell – HT 29 cell monolayer contact to assess resuscitation of VBNC cells

Trypsinized HT-29 cells (2 × 10^5) were deposited in 24-well tissue culture plates (Falcon) for 4 days to obtain almost confluent monolayers [36] and then in antibiotic-free medium for 24 h. HT-29 cell monolayers were infected with VBNC cells. After 24 h at 37 °C under 5% CO_2, the presence of culturable cells was tested on TSA plates. Cell monolayers were then reincubated in culture medium containing 100 μg/mL gentamicin for 1 h 30 min at 37 °C under 5% CO_2. After washing with PBS (phosphate

buffer saline, pH 7.3), HT-29 cells were directly lysed adding 1 mL cold distilled water at 4 °C. The number of culturable cells released from HT-29 cells was assessed on TSA by appropriate dilutions.

2.4.2. Mouse virulence assays

Six-week-old conventional Swiss female OF1 mice were used (Iffa-credo, Saint–Germain-sur-l'Arbresle, France). They were maintained on sterilized wood shavings with free access to water and sterilized food. The mice used to test culturable cells were infected with 10^3 cells (100 µL of suspensions containing 10^4 culturable cells/mL), the mice used to test VBNC cells were infected with 10^3 metabolically active cells (100 µL of suspensions containing 10^4 active cells/mL). The mice were infected by intravenous inoculation (IV). Five mice were inoculated for each condition.

Culturable bacteria (positive controls) were grown on BHIA slopes overnight at 37 °C. The cultures were standardized turbidimetrically and diluted appropriately in physiologically buffered water. The inocula were resuspended in order to obtain 3 log CFU in 100 µL for tail injection. Each inoculum was verified by determining culturable counts on TSA plates.

No further preparation was performed for VBNC cells, because the bacterial concentration verified by CTC and DVC tests was 10^4 active bacteria/mL of microcosm water for each VBNC strain.

The mice used to test dead cells were inoculated with heat-killed *Listeria* cells, obtained from 10^6 culturable cell/mL suspensions treated by autoclave (121 °C – 15 min). The cellular concentration of these suspensions were higher than the other suspensions, in order to compare with the VBNC suspensions, which contained 10^4 VBNC cells and 10^6 non-viable cells.

Negative control mice were inoculated with 100 µL of sterilized water.

The mice were sacrificed by cervical dislocation 2 days after IV injection. The spleens were removed aseptically. Enumeration of culturable bacteria in the spleen has been demonstrated to provide the most consistent results in the quantitative evaluation of virulence [8]. Moreover, only the IV route produced splenic colonization, the levels of splenic infection obtained being related to the number of bacteria inoculated [2]. The samples were diluted ten fold in physiologically buffered water and plated onto TSA medium. The volume of PBS added depended on the weight of each spleen. The numbers of culturable bacteria were assessed after incubation at 37 °C for 48 h. The average number of cfu per sample was calculated only from positive samples.

In order to detect the presence or absence of *Listeria monocytogenes* strains in spleen mixtures, we used a ListerScreen test kit (AES Laboratoire, Combourg, France). ListerScreen can be used with environmental or food samples. Immunomagnetic Capture (IMC) was carried out by the ListerScreen method, according to the protocol developed by the AES Laboratoire and the Laboratoire de Touraine (Tours, France). After removing samples for plating TSA medium, the remaining spleen mixtures were added to vials containing 100 µL of magnetic beads which were rotated at 6 rpm for 2 h at room temperature. The vials were then placed in a magnetic rack for separation of magnetic beads from the spleen mixtures. The beads were washed twice in capture buffer, and then resuspended in 200 µL of capture buffer. Finally, 10 min centrifugation was performed at 10 000 *g*. The pellets were recuperated and frozen at –20 °C for subsequent PCR.

For PCR, the beads were resuspended in 200 µL of 0.05% PBS Tween Buffer 20 (pH 7.4) (Eurogentec, Belgium) and heated for 5 min at 95 °C. After centrifugation (10 min at 10 000 *g*), 5 µL of the supernatant was subjected to PCR. PCR amplification of a fragment internal to *hly* of 388 bp, the *Listeria monocytogenes* gene coding for listeriolysin O, was performed using primers G0 (5' GAA-TGT-AAA-CTT-CGG-CGC-

Table I. Conditions of incubation and concentration of different VBNC cellular suspensions used in virulence tests.

Strains	Incubation conditions		Concentration of suspensions at inoculation time[a] (cells/mL ± SD*)	
	Temperature (°C)	Time ± SD* (days)[b]	Active cells Count (DVC)	Total cell count
CNL 895807	20	25 ± 4	$4.33 \times 10^4 \pm 0.35$	$6.1 \times 10^6 \pm 0.4$
LO 28	20	27 ± 3	$4.22 \times 10^4 \pm 0.3$	$6.2 \times 10^6 \pm 0.4$
ATCC 19115	20	24 ± 3	$4.4 \times 10^4 \pm 0.4$	$6.1 \times 10^6 \pm 0.3$
Scott A	4	47 ± 4	$4.1 \times 10^4 \pm 0.4$	$6.2 \times 10^6 \pm 0.4$

[a] Inocula at To (suspension in microcosm water) were around about 10^6 cfu/mL.

[b] Time necessary (days) to lose the culturable state.

* SD: standard deviation.

AAT-CAG-3') and D0 (5' GCC-GTC-GAT-GAT-TTG-AAC-TTC-ATC-3'), shown by Bohnert et al. [7] to be specific for *Listeria monocytogenes.* The reaction mixture contained 10× PCR buffer, 2.5 mM of each dNTP, 25 µM of each primer, 5 µL of the DNA preparation and 0.1 U of HotGoldStar DNA polymerase (Eurogentec) in a total volume of 50 µL. Amplification was carried out for 10 min pre-PCR cycle to activate the enzyme and then 35 cycles of 1 min at 95 °C, 1 min at 65 °C and 1 min at 72 °C, and extension at 72 °C for 10 min. Amplified products were detected by electrophoresis in a 1.2% agarose gel in 1× TAE buffer with ethidium bromide.

2.5. Statistical analysis

The differences between averages were calculated by analysis of variance. Probabilities less than or equal to 0.05 were considered significant.

3. RESULTS

3.1. Production of VBNC cells by microcosm water model

Whereas the culturability on the PCA medium regularly decreased and was lost (< 1 cfu/mL) within 4 weeks for CNL 895807, LO 28 and ATCC 19115 strains and 7 weeks for the Scott A strain, the total number of cells detected after DAPI staining by epifluorescence microscopy remained constant. In addition, metabolic activity was detected (10^4 viable cells/mL) by both DVC and CTC staining, regardless of the starvation exposure time (Tab. I). Moreover, passage of VBNC cells on different media such as BHI, peptone water, DMEM, DMEM-FCS did not restore a culturable state. Bacterial cells present in microcosm water were thus considered to be in an Active But Non Culturable state. The VBNC state was maintained over 10 weeks of starvation for each strain tested.

3.2. Virulence assays

3.2.1. Interaction with cells

The in vitro virulence test on HT-29 cells was based on the ability of *Listeria monocytogenes* to form plaques in cell monolayers. For culturable cells, the number of plaques formed showed that the strains ATCC 19115, LO28 and Scott A were virulent. Strain CNL895807 which did not form plaques was considered as avirulent (Tab. II). No plaques were observed for

Table II. Virulence of VBNC and culturable cells of *Listeria monocytogenes* assessed in vitro.

Strains	Physiological state	Plaque forming assay[a] Mean (log ± SD)
Scott A	Culturable	6.55 ± 0.30
	VBNC	0
CNL 895807	Culturable	0
	VBNC	0
LO 28	Culturable	5.56 ± 0.06
	VBNC	0
ATCC 19115	Culturable	6.59 ± 0.04
	VBNC	0

[a] Number of plaques for 4 log cfu deposited. The results correspond to the mean ± standard deviation (SD) of the logarithmic values from two independent experiments performed in duplicate.

Table III. Virulence of VBNC and culturable cells of *Listeria monocytogenes* assessed in vivo.

| Strains	Physiological state	Intravenous test (spleens)[b] Mean (log ± SD)	Number of positive cultures (spleen)
Scott A	Culturable	5.52 ± 0.38	5/5
	VBNC	0	0/5
	HKC[a]	0	0/5
CNL 895807	Culturable	4.16 ± 0.63	5/5
	VBNC	0	0/5
	HKC	0	0/5
LO 28	Culturable	5.63 ± 0.31	5/5
	VBNC	0	0/5
	HKC	0	0/5
ATCC 19115	Culturable	5.52 ± 0.56	5/5
	VBNC	0	0/5
	HKC	0	0/5
Negative control		0	0/5

[a] HKC: heat killed cells.

[b] Number of bacteria per spleen homogenate. The results correspond to the mean ± SD of logarithmic values from contaminated mice.

VBNC cells, regardless of the strain tested. Thus, VBNC cells seemed to be unable to enter the cell monolayer and were classified as avirulent.

After 24 h contact no *Listeria* were recovered in the supernatants or in HT-29 cells. These findings show that contact with HT29 cells prevented resuscitation of VBNC cells.

3.2.2. Virulence assays in mice

As demonstrated by spread plate count, spleen mixtures were infected with culturable bacteria for all strains used (Tab. III). According to the results obtained by the Plaque Forming Assay, strain CNL895807 seemed to be less virulent than the other strains tested. With the IMC-PCR method
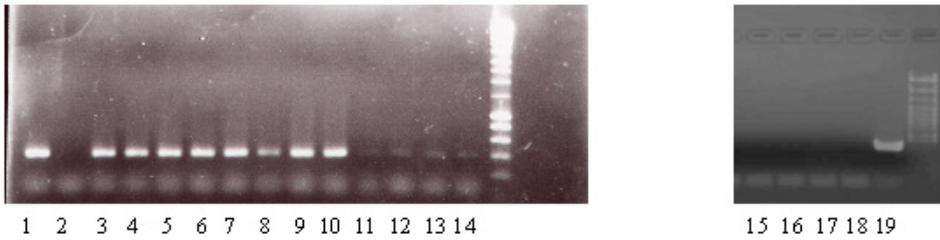
Figure 1. IMC-PCR applied on spleen samples 48 h after IV injection. Lane 1: positive control; lane 2: negative control; lanes 3 to 6: positive control for strains Scott A (3), LO28 (4), CNL 895807 (5), ATCC 19115 (6); lanes 7 to 10: sample of spleens of mice after IV injection of culturable strains Scott A (7), LO28 (8), CNL 895807 (9), ATCC 16115 (10); lanes 11 to 14: sample of spleens of mice after IV injection of VBNC strains Scott A (11), LO28 (12), CNL 895807 (13), ATCC 16115 (14); lanes 15 to 18: sample of spleens of mice after IV injection of heat killed strains ScottA (15), LO28 (16), CNL 895807 (17), ATCC 16115 (18); lane 19: sample of spleens of mice after IV injection of culturable Scott A strain.

(Fig. 1), a strong signal was observed in all samples of spleen infected by culturable cells.

After 48 h of incubation at 37 °C, no colonies were observed on the TSA plates spread with spleen mixtures from mice infected with VBNC cells or from dead cell control mice or negative control mice (Tab. III). The presence of VBNC cells in the spleen mixture was also evaluated by the IMC-PCR method. Amplification of a 388 bp fragment revealed the presence of *Listeria monocytogenes* DNA in spleen mixtures after IV injection of VBNC cells (Fig. 1). The samples infected by heat-killed *Listeria* tested by PCR did not show any signals.

The spleens of mice infected with VBNC cells therefore did not contain any culturable cells. On the contrary, the weak signal revealed by PCR in samples from the same mouse spleens showed the presence of *Listeria monocytogenes* cells. These results prove that these cells were present in mouse spleens but did not return to a culturable state. VBNC cells cannot be regarded as virulent according to this animal model, but neither can they be regarded as dead cells.

4. DISCUSSION

The aim of this study was to investigate whether VBNC *Listeria monocytogenes* cells remained virulent. Two markers of virulence were chosen: i.e. the ability to colonize the mouse spleens and the ability to form plaques within an enterocyte cell line monolayer. *Listeria monocytogenes* has been reported to enter the VBNC state and to remain as such for extended periods in microcosms, depending on a variety of factors such as temperature and salinity [4, 6]. In this study, LO 28, ATCC 19115 and CNL 895807 strains of *Listeria monocytogenes* became VBNC when incubated in a microcosm water system at 20 °C, and for the Scott A strain at 4 °C. In this state, the cells were unable to grow in usual culture medium and could not be resuscitated by traditional resuscitation techniques, but they retained metabolic activity (10^4 active cells/mL). Different explanations have been suggested for the acute loss of culturability with starvation. Theunissen et al. [34] suggested possible membrane damage due to the partial removal of proteins from the outer membrane.

In contrast to culturable cells, no VBNC cells were recovered from mouse spleens. These findings showed that VBNC cells seem to be avirulent in the conditions tested in the present study. These results agree with previous studies, showing that VBNC cells are generally avirulent. Rahman et al. [26] reported that copper-stressed VBNC

Aeromonas hydrophila cells showed reduced virulence compared to control cells. The same results were established for *Vibrio vulnificus* [18, 22] and *Francisella tularensis* [15]. However, some studies have demonstrated that VBNC cells retain some pathogenic effects [23–25]. Some authors have shown that *E. coli* VBNC cells retain their pathogenicity by keeping the ability to produce enterotoxin [3, 23], or by preserving plasmids involved in pathogenicity [9]. Oliver and Bockian [22] showed that injecting *Vibrio vulnificus* VBNC cells in mice killed the animals. In contrast VBNC cells of *Listeria monocytogenes* seemed to present no risk in mice in the present study.

In the invasion assay, culturable cells adhered to HT-29 cells throughout the entire period of observation, in contrast to VBNC cells, which did not adhere and spread to HT-29 cells. The virulence proteins in VBNC *Listeria monocytogenes* cells, which are necessary to express the virulence mechanism, therefore did not seem to be synthesized. The concept that bacterial adhesion to tissues is a prerequisite for infection is well accepted [31], especially for pathogens that invade human hosts like *Listeria monocytogenes*. There have been no reports to date that indicate maintenance of adhesive property by VBNC organisms, except for one study performed by Rahman et al. [25] on *Shigella dysenteriae* Type 1. They demonstrated that VBNC *Shigella dysenteriae* type 1 remains potentially virulent, on the basis of the experimental evidence that maintained the production of the ShT toxin and adherence to Henle 407 cells.

In a number of studies which demonstrate that VBNC cells retain virulence, it is suspected that cells revert to a culturable state [13, 33]. It is difficult in animal model experiments to appreciate whether the lethal effect is due to VBNC cells or to their resuscitation. The physiological characteristic most commonly associated with pathogenicity is culturability, regardless of cellular metabolic activity. Using several reliable methodologies and fluorescent dyes to characterize the metabolic activity of the cell population, we demonstrate that with these particular stress factors, pathogenicity was lost concomitantly with the ability of the cells to multiply (culturability), whereas cell metabolic activity remained undamaged according to respiratory activity (CTC) and elongation (DVC). The simultaneous loss of culturability and pathogenicity has also been observed in *Campylobacter jejuni*, despite a high level of metabolic activity in the cell population tested [11, 20]. Using a *Salmonella typhimurium* model, Caro et al. [12] showed that when exposed to starvation in drastic conditions (artificial sea water and UVc radiation) this bacterium loses its virulence in a manner concomitant with the loss of culturability. In the VBNC state, *Campylobacter jejuni* loses their adhesion capacity, and hence their enteropathogenicity, although after resuscitation the associative index on HeLa cells was restored to almost a baseline level [10, 11]. Consequently, we cannot say whether the loss of pathogenicity is permanent or transient. In almost all published studies, resuscitation allowed bacteria to recover virulence. In this experiment, VBNC cells were found to be avirulent because of the lack of resuscitation. The models used here were probably inappropriate to resuscitation of VBNC cells.

Our results showed that VBNC *Listeria monocytogenes* cells did not resuscitate after IV injection in mice, as demonstrated by the culturable method. However, the rapid immunomagnetic capture PCR method proved that cells were located in the spleen after inoculation of VBNC cells. The absence of a signal in dead cell controls obtained with killed *Listeria* cells showed that the weak signal obtained with VBNC cells is not simply the route of clearance of bacterial cells injected into the blood stream of these animals.

Our findings cast serious doubts on the importance attributed to the viable but nonculturable state. Further studies should characterize cell viability and investigate

whether *Listeria monocytogenes* VBNC cells can be resuscitated and regain their virulence.

REFERENCES

[1] Anonymous, Update: Multistate outbreak of listeriosis-United States, 1998–1999, Morb. Mortal. Wkly. Rep. 47 (1999) 1117–1118.

[2] Audurier A., Pardon P., Marly J., Lantier F., Experimental infection of mice with *Listeria monocytogenes* and *Listeria innocua*, Ann. Microbiol. (Paris) 131B (1980) 47–57.

[3] Barcina I., Gonzalez J.M., Iriberri J., Egea L., Survival strategy of *Escherichia coli* and *Enterococcus faecalis* in illuminated fresch and marine systems, J. Appl. Bacteriol. 73 (1990) 229–236.

[4] Besnard V., Federighi M., Cappelier J.M., Evidence of VBNC state in *Listeria monocytogene*s by DVC and CTC-DAPI double staining, Food Microbiol. 17 (2000) 697–704.

[5] Besnard V., Federighi M., Cappelier J.M., Development of a direct viable count procedure for the investigation of VBNC state in *Listeria monocytogenes*, Lett. Appl. Microbiol. 30 (2000) 1–6.

[6] Besnard V., Federighi M., Declerq E., Jugiau F., Cappelier J.M., Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*, Vet. Res. 33 (2002) 359–370.

[7] Bohnert M., Dilasser F., Dalet C., Mengaud J., Cossart P., Use of specific oligonucleotides for direct enumeration of *Listeria monocytogenes* in food samples by colony hybridization and rapid detection by PCR, Res. Microbiol. 143 (1992) 271–280.

[8] Brosch R., Catimel B., Milon G., Buchrieser C., Vindel E., Rocourt J., Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompent mice and its association with typing characteristics, J. Food Prot. 56 (1993) 296–301.

[9] Byrd J.J., Colwell R.R., Maintenance of plasmids pBR322 and pUC8 in nonculturable *Escherichia coli* in the marine environment, Appl. Environ. Microbiol. 56 (1990) 2104–2107.

[10] Cappelier J.M., Magras C., Jouve J.L., Federighi M., Recovery of viable but non-culturable *Campylobacter jejuni* cells in two model, Food Microbiol. 16 (1999) 375–383.

[11] Cappelier J.M., Minet J., Magras C., Colwell R.R., Federighi M., Viable but non culturable *Campylobacter jejuni* cells recover culturability and adhesion abilities after embryonated egg passage, Appl. Environ. Microbiol. 65 (1999) 5154–5157.

[12] Caro A., Got P., Lesne J., Binard S., Baleux B., Viability and virulence of experimentally stressed nonculturable *Salmonella typhimurium*, Appl. Environ. Microbiol. 65 (1999) 3229–3232.

[13] Colwell R.R., Brayton P., Herrington D., Tall B., Huq A., Levine M.M., Viable but nonculturable *Vibrio cholerae* 01 revert to a cultivable state in the human intestine, World J. Microbiol. Biotechnol. 12 (1996) 28–31.

[14] Farber J.M., Peterkin P.I., *Listeria monocytogenes*, a food-borne pathogen, Microbiol. Rev. 55 (1991) 476–511.

[15] Forsman M., Henningson E.W., Larsson E., Johansson T., Sandström G., *Francisella tularensis* does not manifest virulence in Viable but Non-Culturable state, FEMS Microbiol. Ecol. 31 (2000) 217–224.

[16] Jones E.M., Mac Gowan A.P., Antimicrobial chemotherapy of human infection due to *Listeria monocytogenes*, Eur. J. Clin. Microbiol. Infect. Dis. 14 (1995) 165–175.

[17] Kogure K., Simidu U., Taga N., A tentative direct microscopic method for counting living marine bacteria, Can. J. Microbiol. 25 (1979) 415–420.

[18] Linder K., Oliver J.D., Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*, Appl. Environ. Microbiol. 55 (1989) 2837–2842.

[19] Lorber B., Clinical listeriosis-implications for pathogenesis, in: Miller A.J., Smith J.L., Somkuit G.A. (Eds.), Food borne Listeriosis, Elsevier Science, New York, 1990, pp. 41–49.

[20] Medema G.J., Schets F.M., Van de Giessen A.W., Havelaar A.H., Lack of colonization of 1 day old chicks by viable, non culturable *Campylobacter jejuni*, J. Appl. Bacteriol. 72 (1992) 512–516.

[21] Oliver J.D., Formation of Viable But Nonculturable cells, in: Kjelleberg S. (Ed.), Starvation

in Bacteria, Plenum, New York, 1993, pp. 239–272.

[22] Oliver J.D., Bockian R., In vivo resuscitation, and virulence towards mice of viable but nonculturable cells of *Vibrio vulnificus*, Appl. Environ. Microbiol. 61 (1995) 2620–2623.

[23] Pommepuy M., Butin M., Derrien A., Gourmelon M., Colwell R.R., Cormier M., Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight, Appl. Environ. Microbiol. 62 (1996) 4621–4626.

[24] Rahman I., Shahamat M., Kirchman A., Russek-Cohen E., Colwell R.R., Methionine uptake and cytopathogenicity of viable but non-culturable *Shigella dysenteriae* type 1, Appl. Environ. Microbiol. 60 (1994) 3573–3578.

[25] Rahman I., Shahamat M., Chowdhury M.A.R., Colwell R.R., Potential virulence of Viable But Non Culturable *Shigella dysenteriae* Type 1, Appl. Environ. Microbiol. 62 (1996) 115–120.

[26] Rahman M.H., Suzuki S., Kawai K., Formation of viable but non-culturable state (VBNC) of *Aeromonas hydrophila* and its virulence in goldfish, *Carassius auratus*, Microbiol. Res. 156 (2001) 103–106.

[27] Rodriguez G.G., Phipps D., Ishiguro K., Ridgway H.F., Use of a fluorescent redox probe for direct visualization of actively respiring bacteria, Appl. Environ. Microbiol. 58 (1992) 1801–1808.

[28] Rollins D.M., Colwell R.R., Viable but non culturable stage *of Camplylobacter jejuni* and its role in survival in the aquatic environment, Appl. Environ. Microbiol. 52 (1986) 531–558.

[29] Roszak D.B., Colwell R.R., Survival strategies of bacterial in the natural environment, Microbiol. Rev. 51 (1987) 365–379.

[30] Roth W.G., Leckie M.P., Dietzler D.N., Restoration of colony-forming activity in osmotically stressed *Escherichia coli* by betaïne, Appl. Environ. Microbiol. 54 (1988) 3142–3146.

[31] Sansonetti J.P., Physiopathology of intestinal *Salmonella* infection, Rev. Prat. 42 (1992) 2263–2267.

[32] Schlech W.F., Lavigne P.M., Bortulussi R.A., Allen A.C., Haldane E.V., Wort A.J., Hightower A.W., Johnson S.E., King S.H., Nicholls E.S., Broome C.V., Epidemic listeriosis: evidence for transmission by food, N. Engl. J. Med. 308 (1983) 15–24.

[33] Steinert M., Emody L., Amann R., Hacker J., Resuscitation of viable but non culturable *Legionella pneumophila Philadelphia JR32* by *Acanthamoeba castellanii*, Appl. Environ. Microbiol. 63 (1997) 2047–2053.

[34] Theunissen H.J.H., Lemmens-den Toom N.A., Burggraaf A., Stolz E., Michel M.F., Influence of temperature and relative humidity on the survival of *Chlamydia pneumoniae* in aerosols, Appl. Environ. Microbiol. 59 (1993) 2589–2593.

[35] Van Langendonck N., Bottreau E., Bailly S., Tabouret M., Marly J., Pardon P., Velge P., Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains, J. Appl. Microbiol. 85 (1998) 337–342.

[36] Velge P., Bottreau E., Van lanfendonck N., Kaeffer B., Cell proliferation enhances entry of *Listeria monocytogenes* into intestinal epithelial cells by two proliferation-dependent entry pathways, J. Med. Microbiol. 46 (1997) 681–692.

[37] Whitesides M.D., Oliver J.D., Resuscitation of *Vibrio vulnificus* from the viable but non culturable state, Appl. Environ. Microbiol. 63 (1997) 1002–1005.