Investigation on the Intracellular Survival of Bacteria in Free-Living Amoebae

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Abstract

Intracellular survival of bacteria in free-living amoebae was investigated after axenic cultivation of the latter. Amoebae were isolated from sewage and pondwater samples after exposure to bacteriocidal concentration of gentamicin. The amoebae cells were sonicated to release any intracellular bacteria and the bacteria isolated subsequently were challenged with the same concentrations of gentamicin to which the original samples were exposed. Bacteria were seen to survive intracellularly in free-living amoebae after the exposure of the amoebae to the antibiotic gentamicin. With the exception of one Pseudomonas sp isolate, all the bacteria isolated which include species of Pseudomonas, Bacillus, Salmonella, Corynebacterium and Vibrio were susceptible to 10 mg gentamicin. This indicates that some species of bacteria do not only survive intracellularly in amoebae but are also afforded protection against some bacteriocides. We therefore propose that free-living amoebae may not only act as reservoirs but also as vehicles for the transmission of (pathogenic) bacteria.

Introduction

Amoebae are free-living organisms found in water, moist soil, sewage and decaying matter. They feed on bacteria (Miyamoto *et al.*1996). Studies (Danso and Alexander, 1975; Weekers *et al.*, 1993) have however shown that not all bacteria are suitable food sources for amoebae since they survive grazing by amoebae. *Legionella pneumophila* has been known to infect and multiply within some species of free-living amoebae (Rowbotham, 1980). Alexander (1981) also reported that some Gram negative bacteria are able to survive grazing by amoebae.

Bacteria internalized by amoebae may be given unique protection when the protozoa form cysts (Barker and Brown, 1994). King *et al.*, (1988) reported that internalization of coliform bacteria by protozoa gives them protection against external antagonists. Goshko *et al.*, (1983) and Hudson *et al.*, (1983) reported that *Salmonella typhimrium* and *Shigella sonnei* survived ingestion by laboratory strains of *Acanthamoeba castellanii* (amoebae) and *Tetrahyma pyriformis* (ciliates) and were shielded from the activity of free chlorine. The organisms were cultured from chlorine-treated protozoans well after the time required for the inactivation of extracellular cells. Thus organisms trapped within amoebae could be responsible for the persistence of coliform bacteria in chlorine-treated water. This may be of considerable importance in the maintenance of infectious coliform agents in the environment.

The possible role of free-living amoebae in the survival and distribution of (pathogenic) bacteria has received limited attention (Barker and Brown, 1994). This paper describes intracellular survival of bacteria in free-living amoebae isolated from sewage and other natural environments.

Materials and Methods

Collection and Preparation of samples: Ten sewage samples (sw) (from different points of the sewage treatment plant) and ten pond water (pw) samples (from different sources) were collected within University of Nigeria Nsukka, Nigeria. The samples were allowed to stand for 6hr and decanted. Presence of amoebae in test samples was confirmed by simple microscopy. Samples were subsequently treated with gentamicin at 20μg/ml (final concentration) and incubated for 24hr. After incubation, aliquots were examined for viable bacteria cells by microscopic examination of methylene blue smears. The bacteria-free samples were centrifuged (1, 240 xg) for 5min and the supernatant discarded. Filtered and autoclaved water samples were exposed to air and used as controls.

Bacterial bait preparation: The migration of amoebae over a sterile agar surface is sufficient to rid the amoebae of (contaminating) bactria. The method achieves axenic cultivation of amoebae and is induced by the presence of a suitable bait (Neff,1958;King *et al.*,1988). *Escherichia coli* obtained from the Clinical Laboratory of the Department of Microbiology, University of Nigeria,Nsukka Nigeria was used as a bait for the amoebae in this work. They were maintained on nutrient agar slants and stored in the refrigerator until needed. Bait cells were prepared by subculturing from the refrigerated slants into nutrient broth tubes and incubating for 24hr. The resultant growths were centrifuged (4000 xg) for 15min and the supernatant discarded. The bacterial sediment was washed three times by centrifugation using sterile normal saline. The cells were resuspended in normal saline (diluted to a turbidity equivalent to 0.5 Mcforland standard using spectrophotometry) (Spectronic 20 – Bausch and Lomb, Rochester NY)]. This was autoclaved at 121^{0}C for 15 minutes.

Isolation of amoebae: Amoebae were isolated from the samples' sediments using the migration method of Howard (1987). The autoclaved cells of *Escherichia coli* were used as a food source and bait for the amoebae (Neff, 1957). Four loop-fulls of the dead bacterial cells in normal saline were placed on the surface of a Page's amoebae saline (containing 1.5.%` agar) (Fleck and Moody, 1988)

around the periphery. Two loop-fulls of each sediment were placed in the centre with a loop-full of the *E. coli* dead cells. Amoebae migrated out from the central drop and entered the bacterial lawn containing dead cells by the second day after incubation (as revealed by the formation of plaques). Amoebae cells were gently scraped from the surface of the agar and resuspended in sterile Page's saline according to the method of O'Dell (1979). The suspension was washed three times by centrifugation (1,240 xg for 5 min) in sterile saline and kept ready for sonication. This process was repeated for all the samples analyzed.

Sonication of amoebae and Isolation of intracellular bacteria: Washed suspensions were examined microscopically for viable amoebae cells. The cells were then disrupted under ice for 3 min. at 60 KHz using a VIRSONIC CELL DISRUPTER (Model 16 – 850)(VIRTIS COMPANY N.Y. 12525). After that the sonicated sediments were transferred into sterile centrifuge tubes and centrifuged (4000 X g) for 15 min. The sediments were then streaked out on sterile nutrient and MacConkey agar plates. Incubation was done for 24 hr. As controls,pond and sewage water samples were filtered and autoclaved to make them amoebae-free.They were subsequently exposed to air to be recontaminated by bacteria,and examined microscopically to ensure that they are amoebae-free and then subjected to the same treatment as the analyte samples and plated out also on sterile nutrient and MacConkey agar plates. Colony units formed were purified and identified based on morphological and biochemical characteristics according to the criteria of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984; Sneath *et al* 1986).

Testing of antibiotic sensitivity pattern of bacterial isolates: Using the procedure of Bauer *et al* (1996) with Mueller-Hinton agar, the bacterial isolates were assayed against 10μg gentamicin disks. Resistant ones were further exposed to 20μg/ml concentration of the same antibiotic.

Results

The sonicated amoebae isolates yielded, to varying percentages *Bacillus* sp, *Pseudomonas* sp, *Salmonella* sp, *Corynebacterium* sp and *Vibrio* sp. (Table 1) *Corynebacterium* sp were isolated from 60 per cent of the sewage samples tested and from 20 per cent of the pond water samples. *Bacillus* sp occurred next in frequency to *Corynebacterium* sp having been isolated from 50 and 30 per cent of sewage and pond water amoebae isolates respectively.The amoebae-free control samples did not yield any bacterial growth after the sonication process.

All except one *Pseudomonas* sp isolate were susceptible to the 10μg gentamicin disk. When exposed to 20μg /ml concentration of gentamicin however, the resistant *Pseudomonas* showed susceptibility.

Table 1: Percentage occurrence of Bacteria in Sewage and Pond water amoebae isolates

Bacteria isolate	Source of amoebae	% Occurrence
Bacillus sp	Sewage	50
Pesudomonas sp	Sewage	30
Salmonella sp	Sewage	20
Corynebacterium sp	Sewage	60
Vibrio sp	Sewage	10
Bacillus sp	Pond water	30
Pseudononas sp	Pond water	10
Corynebacterium sp	Pond water	20

Discussion

Antibiotic (gentamicin) was applied to the sewage and water samples in this work for two reasons. First was to ensure axenic cultivation of amoebae and by extension, ensure that any bacteria isolated came intracellularly from the amoebae isolates. Secondly it was used as an experimental treatment to check the possible protection given to intracellularly surviving bacteria from antibiotics. Our results have shown that some bacteria survived intracellularly in amoebae for the period between when we exposed the samples to (lethal doses) broad spectrum antibiotic-gentamicin and when the bacteria were eventually isolated from the sonicated sediments of amoebae cells (ca. 68 hr). The recovery of these bacteria and their susuptibility to gentamicin (10μg) disks subsequently shows that their survival was only possible because the amoebae 'hosts' afforded them protection from the antibiotic with which they were challenged. This is in consonance with similar reports (King *et al.*, 1988; Barker and Brown, 1994; Wadowsky *et al.*, 1988; fields *et al.*, 1989) indicating nitracellular survival of bacteria in protozoa albeit in water samples treated with chlorine. Barker and Brown (1994) propounded that intra-protozoal growth of *Legionellae*, sp for example, is a primary mechanism for the survival and multiplication of the bacterium in natural habitats and that *Legionellae* are not simply and only free-living bacteria *per se* but have a highly evolved host/parasite relationship described as protozootic for their survival in natural ecosystems.

The results indicated strongly that amoebae allow survival and possibly enhance the distribution of some species of pathogenic bacteria in the natural environment. The intracellular niche affords protection against adverse environmental conditions and treatment with biocides as evidenced by the exposure of our samples to bacteriocidal concentrations of gentamicin.

This intra-amoebae growth of bacteria as earlier suggested (Barker *et al.*, 1993) may include phenotypes that will be considerably different from *in vitro* grown strains in terms of physiological status, survival and infectively. Changes in the molecular composition of intra-amoebal-grown bacteria could be important in infection process in human host since surface molecules play a vital role in bacterial survival and virulence (Brown and Williams 1985).

Isolation of *Salmonella* sp, *vibrio* sp, *Bacillus* sp, *Corynebacterium* sp and *Pseudomonas*

sp from amoebae cells in this investigation is in line with what other researchers working with laboratory- induced amoebic grazing of bacteria discovered i.e that different bacteria survive intracellularly in some and different protozoa (King et al.,1988). This portends a public health problem especially in our environment where much sanitary weight is put on biostatics and biocides as a means of water purification. The ability of amoebae to survive, for example chlorine treatment (King et al.,1988) and by extension ensuring the survival of (pathogenic) bacteria intra-cellularly will definitely have environmental and consequent clinical implications for a society such as ours in which human and industrial waste management and disposal leave much to chance. There is the possibility that amoebae may be vehicles for the transmission of some bacteria infections endemic in our society today but which have obscure modes of transmission and survival. It is recommended therefore that more attention should be given to this area of our public health life by health workers, medical personnel and environmentalists.

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