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Brief report

# Amoebae promote persistence of epidemic strains of MRSA

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## Summary

**The control of healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA) infection is of concern worldwide. Given the evidence that several pathogenic species replicate within amoebae and emerge more virulent and more resistant and the abundance of amoebae in healthcare settings, we investigated interactions of *Acanthamoeba polyphaga* with epidemic MRSA isolates. MRSA proliferated in the presence of amoebae, attributable partly to intracellular replication. Following 24 h of co-culture, confocal microscopy revealed that *c.* 50% amoebae had viable MRSA within phago-lysosomes and 2% of amoebae were heavily infected with viable cocci throughout the cytoplasm. Infection control strategies should recognize the contribution of protozoa.**

## Introduction

The control of healthcare-associated infection is of concern worldwide and particular attention is focused on the commonest multi-resistance bacterium, methicillin-resistant *Staphylococcus aureus* (MRSA). Recently released figures show that infections caused by MRSA rose 5% between 2003 and 2004 and mortality rates increased 15-fold between 1993 and 2002 (White, 2004). Within the UK, these figures have prompted an urgent review of strategies to control healthcare-associated infection, including an emphasis on clean hospital environments and good hygiene practice (Department of Health, 2003).

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While MRSA is typically spread through skin contact, it can survive on inanimate objects within hospital wards (Oie *et al.*, 2005). Amoebae also commonly inhabit the same areas within healthcare environments (Rohr *et al.*, 1998). While many bacterial species are digested by amoebae, some pathogenic bacteria can survive and replicate intracellularly; the prototypical example being *Legionella pneumophila* (Rowbotham, 1980; 1983; Barbaree *et al.*, 1986; Brown and Barker, 1999; Molmeret *et al.*, 2005). Crucially, *L. pneumophila* released from amoebae are less susceptible to biocides and antimicrobials (Kilvington and Price, 1990; Barker *et al.*, 1992; 1995) and are more invasive than their free-growing counterparts (Cirillo *et al.*, 1994). Furthermore, amoebal cysts can trap pathogens and thus potentially disperse them widely by air currents (Kilvington and Price, 1990). As effective control of MRSA within healthcare environments requires better understanding of their ecology, we studied the interactions of epidemic MRSA isolates 3, 15, 16, Berlin 825/96 and *Escherichia coli* K-12, the latter a common food source for amoebae, alongside the ubiquitous amoeba, *Acanthamoeba polyphaga*.

## Results and discussion

Following co-culture at a multiplicity of infection (moi) of one amoeba: 100 bacteria and a temperature of 37°C, extracellular EMRSA-3, 15 and 16 numbers increased significantly ($P < 0.01$) in the presence of *A. polyphaga* compared with control bacteria after 24 h (Fig. 1A). There was little bacterial growth in media filtered after co-culture (data not shown). Consequently, the increase in extracellular counts was largely the result of released intracellularly grown bacteria and not cryptic growth on amoebal products. Importantly, extracellular counts of all EMRSA strains were 1000-fold greater in the presence of amoebae compared with that in their absence. Extracellular numbers of a laboratory strain of *E. coli* K-12, decreased significantly ($P < 0.01$) during the initial 24 h compared with control bacteria (Fig. 1A), and similar differences were still evident following 6 days of co-culture (data not shown). Amoebal counts were reduced after 24 h in the presence of EMRSA-15 and -16, probably releasing EMRSA. However, the reduction in number was not sig-

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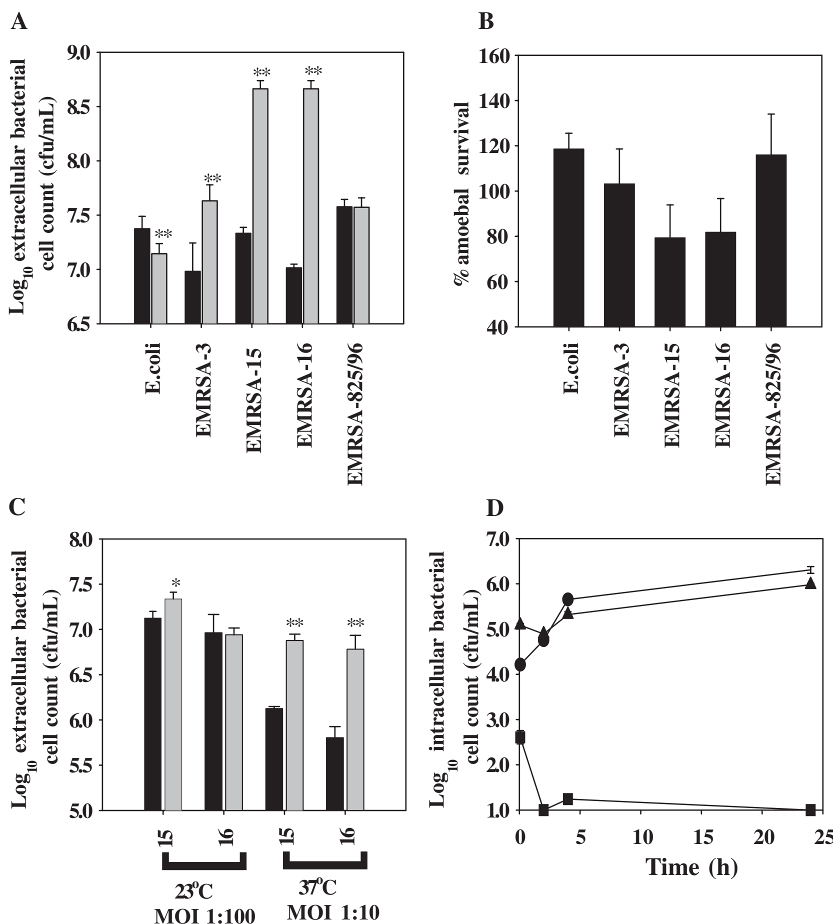


**Fig. 1.** A. Extracellular bacterial counts following 24 h in the presence (grey bars) or absence (black bars) of *A. polyphaga* (moi 1:100, 37°C).
B. Survival of *A. polyphaga* following 24 h in the presence of bacteria (moi 1:100, 37°C).
C. The effects of a temperature of 23°C (moi 1:100) and 37°C (moi 1:10) on extracellular bacterial counts of EMRSA-15 and 16 following 24 h in the presence (grey bars) or absence (black bars) of *A. polyphaga*.
D. Intracellular growth kinetics of EMRSA-15 (▲), EMRSA-16 (●), and *E. coli* K-12 (■) within *A. polyphaga*. Data represent at least $n = 5 \pm$ standard error of the mean ($*P < 0.05$, $**P < 0.01$). All co-cultures were carried out in 12 well plates in Neff's amoebal saline (Page, 1976), and washed stationary-phase amoebae were allowed to adhere for 30 min before adding washed stationary-phase bacteria. Bacterial numbers were monitored on Luria–Bertani (LB) agar incubated overnight at 37°C. Amoebal viability was monitored using the trypan blue exclusion assay. T-tests were employed after normality was ascertained. Intracellular survival kinetics of EMRSA-15, -16, and *E. coli* K-12 were monitored by preparing co-cultures as described and plates were centrifuged to increase contact. After 2 h of incubation, the attached amoebal layer was washed twice before addition of lysostaphin (20 mg $l^{-1}$ for 2 h) or gentamicin sulfate (100 mg $l^{-1}$ for 1 h), to kill over 99.99% of extracellular bacteria. Antimicrobials were removed by washing twice (designated time 0 h), and at time intervals thereafter, extracellular (released bacteria) and intracellular bacterial numbers were monitored and combined to give an indication of intracellular survival (Gao and Kwaik, 2000).

nificantly different compared with amoebae without bacteria (Fig. 1B). When the co-culture incubation temperature was reduced to 23°C (moi 1:100), the increase in survival was marginal (EMRSA-15) or not seen (EMRSA-16) (Fig. 1C). Intracellular growth kinetics were consequently not followed at 23°C. At 37°C, the increase in survival (EMRSA 15 and 16), however, remained significant when the moi was reduced to 1:10. Intracellular growth kinetics revealed that EMRSA-15 and -16 replicated approximately 10- and 100-fold, respectively, after 24 h. In contrast, *E. coli* K-12 did not proliferate and was not detectable after 2 h (Fig. 1D).

Fluorescent confocal microscopy revealed that after 24 h of co-culture, *c.* 50% of amoebae contained viable EMRSA-16 (Fig. 2A–D) within phago-lysosomes, and *c.* 2% of amoebae had viable bacteria throughout the cytoplasm (Fig. 2E–H). So, EMRSA-16 may be able to withstand acidic conditions and accomplish intracellular replication. The heavily infected amoebae also stain red throughout the cell providing evidence that perhaps phago-lysosomal fusion does occur before the bacterium escapes the phago-lysosome, thus also releasing the red stain to the cytoplasm.

The present study is the first to demonstrate that epidemic MRSA isolates proliferate in the presence of *A. polyphaga*, due at least partly to intracellular replication. Similar to *L. pneumophila* survival within the ciliate *Tetrahymena pyriformis*, proliferation was greater at 37°C than at 23°C (Fields *et al.*, 1984). Confocal fluorescent microscopy revealed that food vacuoles may have undergone phago-lysosomal fusion. However, recent data investigating *Burkholderia cepacia* complex within *A. polyphaga* showed their intracellular survival through an alternative mechanism of survival, specifically within an acidified vacuole that was distinct from the lysosomal compartment (Lamothe *et al.*, 2004). We cannot rule out this mechanism of intracellular survival for MRSA. Invasion assays have shown that uptake of *S. aureus* by mammalian cells involves fibronectin binding protein (Heilmann *et al.*, 2005). Although a similar mechanism may occur with amoebae, we do not know whether uptake is active or due to phagocytosis alone. Regardless, evidence suggests that *S. aureus* is killed rapidly at pH 2, but acid resistance can be developed if first adapted via a *sigB*-mediated pathway (Cotter and Hill, 2003). Also, *S. aureus* can comfortably tolerate mildly acidic conditions, such as

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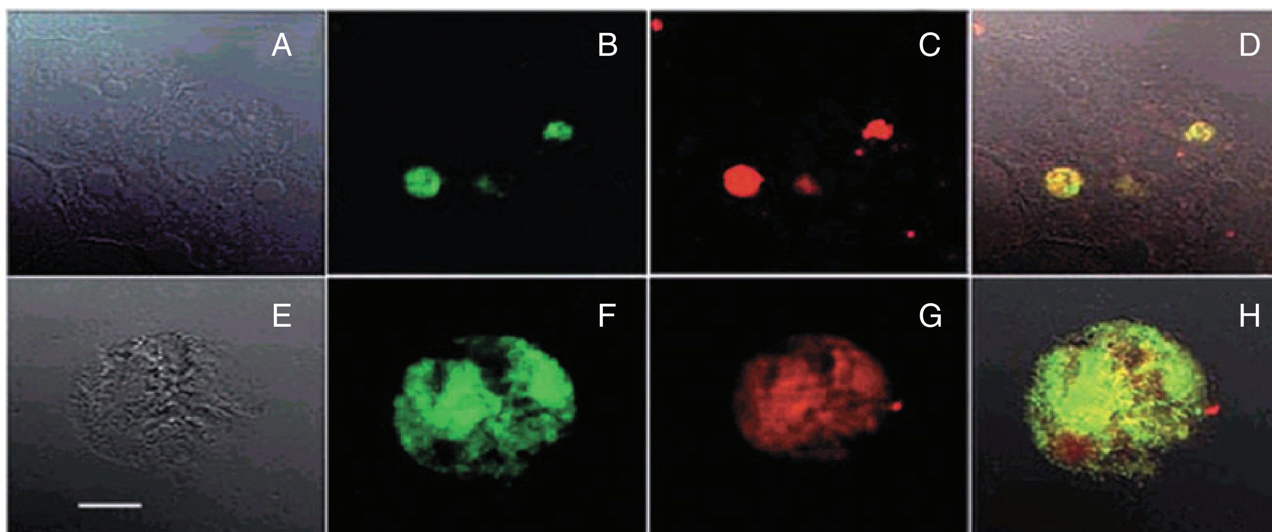


**Fig. 2.** Confocal microscopy of EMRSA-16 within phago-lysosomes (A–D, depth 1.06 μm) and throughout the cytoplasm of (E–H; depth 1.63 μm) *A. polyphaga*. EMRSA-16 are shown green (B and F), acidic organelles are red (C and G) and the corresponding overlay (D and H). The corresponding light micrographs are shown (A and E). Scale bar = 10 μm. Co-cultures were set up (moi 1:100, 37°C) with prestained EMRSA-16 (10 μM Cell Tracker BIODIPY Viable Green; Molecular Probes, Leiden, the Netherlands). Following 24 h, amoebae were harvested by cell-scraping, and stained with 5 nM LysoTracker Red (Molecular Probes) which stains lysosomes red and can be used as an indicator of phago-lysosomal fusion. Cells were air-dried on poly L-lysine slides and examined under a Zeiss Axiovert 2 confocal microscope (Carl Zeiss vision, Oberkochen, Germany).

those encountered on the skin, and a decline in pH triggers changes in gene expression including in genes associated with virulence factor production and pH homeostasis (Weinrick *et al*., 2004). Survival of *S. aureus* within mammalian cells is instigated by firstly escaping from the endosome, facilitated through utilization of *agr*-regulated exoproteins (Shompole *et al*., 2003). Interestingly, *agr* is a member of the *S. aureus* mild acid stress regulon, being induced upon exposure to pH 5.5. The precise mechanisms that allow the survival of epidemic MRSA strains in *A. polyphaga* remain to be deciphered.

In summary, we have shown that the presence of *A. polyphaga* can cause the proliferation of MRSA isolates. We propose that infection control policies recognize the role of protozoa in the survival of MRSA and indeed of other pathogens, and evaluate control procedures accordingly.

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