

Arsenic Toxicity in Pathogenic *Staphylococcus Epidermidis* and *Klebsiella Pneumoniae*

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Abstract

The sensitivity of pure cultures of *Staphylococcus epidermidis* and *Klebsiella pneumoniae* towards arsenic was studied with particular reference to biochemical changes induced by the heavy metal in these organisms. Arsenic strongly inhibited the growth and viability of both the organisms. Addition of arsenic prolonged the lag phase and this was found to be the concentration dependent phenomenon. The Minimum inhibitory concentration (MIC) determined was 200 ppm and 20 ppm in *S. epidermidis* and *K. pneumoniae* respectively that inhibited growth, synthesis of protein, DNA, RNA completely and activity of dehydrogenases of the TCA cycle. In *S. epidermidis* and *K. pneumoniae*, cell wall, membrane and cytoplasm 24.5%, 32.5%, 43% and 20%, 35%, 45% arsenic respectively got incorporated. As the activity of dehydrogenases was inhibited by arsenic, cells were incapable of oxidizing substrate. It resulted in limited supply of energy rich compounds such as ATP that affected the synthesis of macromolecules. Ultimately multiplication and growth of the organism got ceased.

Keywords: Arsenic toxicity, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, Mechanism, Dehydrogenases

1. Introduction

The transition metal arsenic has a long history of human exploitation as both a poison and a medicine. In more recent times Ehrlich's discovery of the antisyphilitic drug arsephenamine (also known as Salvarsan) by systematic chemical modification of arsenic derivatives marked the beginning of modern pharmaceutical research. Arsenic trioxide is used today in cancer treatment (Evens et al., 2004; Lu et al., 2007; Wang and Chen, 2008).

It was found that exposure to arsenic evoked a broad spectrum of cellular reactions in *Saccharomyces cerevisiae* (Tamas and Wysocki, 2001; Haugen et al., 2004; Thorsen et al., 2007; Jin, 2008) and in higher eukaryotes also (Salviikow and Zhitkovich, 2008). In recent past quite a lot of work has been done with reference to the interaction of metals and microorganisms. Most of the heavy metals have been reported to be toxic to living cells (Abelson and Aldous, 1950; Pickett and Dean, 1979; Babich and Stotzky, 1980; Bagde and Varma, 1982; Bhagwat and Shrivastava, 1993). Toxicity of metal is largely a function of concentration. The toxicity of a metal or metal compound results into cell death or injury including carcinogenic and teratogenic effects (Rao and Viraraghavan, 1991). It may also affect various enzyme systems and metabolic processes of microorganisms (Wimpeny and Cole, 1968; Martinez, et al., 1991; Komen, et al., 1991).

In the present investigation, toxic effect of heavy metal arsenic on pathogenic bacteria *S. epidermidis* and *K. pneumoniae* was studied. Emphasis was given on effect of this metal on various parameters such as growth, synthesis of protein, DNA and RNA, localization of the metal in cell wall, membrane and cytoplasm and synthesis of dehydrogenases enzymes, ultimately suggesting the mode of action of arsenic.

2. Materials and Methods

2.1 Selected Organisms and MIC Determination

In this study, bacterial strains of *Staphylococcus epidermidis* ATCC 12228 and *Klebsiella pneumoniae* ATCC 10031 were obtained from the National Collection of Industrial Microorganisms (NCIM), Pune. Both the organisms were grown at 37°C in a nutrient broth medium (Himedia, India). Experiments were carried out in

Ehrlenmeyer flask of 100 ml capacity with side arm. Culture medium was autoclaved at 121°C for 20 minutes. Incubation was carried out at 37°C for 48 hours on incubator shaker at 100 r.p.m.

Selected organisms, usually grown at 37°C in a nutrient broth medium and maintained at 5°C were subcultured and after 24 hour incubation, used as an inoculum to inoculate experimental flasks with nutrient broth medium. Individual flask was inoculated with 1.00 ml of inoculums prepared as above. The final volume in the flask was 50 ml. Heavy metal arsenic in the form of arsenic trioxide (Himedia, India) used for these experiments was extra pure.

Various concentrations of arsenic were tested for determining MIC of arsenic in two selected microorganisms. After MIC concentrations of arsenic for *Staphylococcus epidermidis* and *Klebsiella pneumoniae* were found out, MIC concentrations and subsequent lower concentrations than MIC were selected for final experimentation. Accordingly, the final concentrations of arsenic tested against *S. epidermidis* were 0, 50, 100, 150 and 200 ppm and 0, 5, 10, 15, 20 against *K. pneumoniae*.

2.2 Estimations for protein, DNA, RNA

Estimations for protein by Folin-Lowry method, DNA by Diphenyl amine (DPA) method and RNA by Orcinol method were made after 6 hourly intervals (Plummer, 1971). Protein reacts with the Folin's reagent to give blue colored complex which was measured at 750nm. On treatment of DNA with DPA, a blue colored compound was formed under an acidic condition which was measured at 595nm. Orcinol method for RNA is a general reaction for pentoses and depends on the formation of furfural when the pentose was heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give green color which was measured at 665nm. The values of protein, DNA and RNA for samples, µg/ml were calculated by referring to standard graph.

2.3 Estimation of arsenic in cell fractions

Sub cellular fractions of cell wall, membrane and cytoplasm were obtained by procedure given by Mitra et al. (1975). Cells of microorganisms *S. epidermidis* and *K. pneumoniae* were treated with arsenic at 200 ppm and 20 ppm respectively for 48 hours and washed and reacted with Tris HCl buffer. EDTA and Lysozyme were used for breaking cell wall. After centrifugation cell wall fraction was removed and pellet was resuspended in Tris HCl buffer and reacted with potassium phosphate buffer and MgSO₄. After centrifugation supernatant was taken as cytoplasm and pellet was used as cell membrane fraction. Arsenic was estimated by Molybdenum Blue Method (Sandel, 1944)

2.4 Dehydrogenases activity

The dehydrogenases activity was assayed as per procedure followed by Guha and Mookerjee (1978). Cells were grown for 48 hours at 37°C and used as sample. To these cells chloramphenicol and MIC concentration of arsenic metal was added. Substrates of TCA cycle were added to each tube. Potassium phosphate buffer and Triphenyl Tetrazolium chloride solution were finally added and OD was measured at 540nm. The OD values were compared with the OD values of the control tubes and percentage inhibition of the activity of enzymes was calculated.

3. Results

Influence of arsenic on the growth of pure culture of *S. epidermidis* and *K. pneumoniae* as determined by the optical density of bacterial suspension after incubation for 48 hours with 6 hourly intervals is shown in Fig. 1 and Fig. 2. The growth of organism decreased with the increase in the concentration of arsenic. Percentage of growth inhibition of arsenic on *Staphylococcus epidermidis* and *Klebsiella pneumoniae* has been given in table 1 and 2 respectively. At 200 ppm arsenic concentration, the growth of organism *S. epidermidis* was completely inhibited while at 20 ppm arsenic concentration growth of *K. pneumoniae* was completely inhibited, hence making these as Minimum Inhibitory Concentrations (MIC). Lag phase was prolonged, log phase was delayed due to the addition of arsenic and hence growth of microorganisms was affected.

Protein contents of the organism (µg/ml) got affected when arsenic was added in the growth medium. 200 ppm and 20 ppm arsenic metal inhibited synthesis of protein completely in *S. epidermidis* and *K. pneumoniae* respectively. Inhibition of protein contents of organisms at different concentrations of arsenic after incubation for 48 hours with 6 hourly intervals is shown in figure 3 and 4 and percentage protein inhibition at different concentrations of arsenic is given in table 1 and 2 for *Staphylococcus epidermidis* and *Klebsiella pneumoniae* respectively.

In Fig. 5 and Fig. 6, RNA contents of the cells at different concentrations of arsenic against *S. epidermidis* and *K. pneumoniae* respectively are shown. 200 ppm and 20 ppm arsenic inhibited the synthesis of RNA completely in

S. epidermidis and *K. pneumoniae* respectively. Percentage inhibition of RNA contents of organisms at different concentrations of arsenic after incubation for 48 hours with 6 hourly intervals is given in tables 1 and 2 for *Staphylococcus epidermidis* and *Klebsiella pneumoniae* respectively.

Arsenic also affected the DNA contents of the cells of the *S. epidermidis* and *K. pneumoniae* (Fig. 7, Fig. 8). Increasing concentrations of arsenic decreased DNA contents of both the organisms. 200 ppm and 20 ppm arsenic metal inhibited synthesis of DNA completely in *S. epidermidis* and *K. pneumoniae* respectively (Tables 1 and 2)

Sub cellular fractions of the cell wall, membrane and cytoplasm of the organisms after treatment with MIC concentration of the metal were obtained after 48 hours (Table 3) and metal was estimated in all fractions separately. In *S. epidermidis* 24.5%, 32.5% and 43% arsenic got localized in cell wall, membrane and cytoplasm respectively and in *K. pneumoniae* 20%, 35% and 45% respectively got localized. When exposed to arsenic, cells of both the organisms showed inhibition of dehydrogenases. Inhibition of dehydrogenases activity in *S. epidermidis* was Glutamic 93.4%, Succinic 84.6%, α -ketoglutaric 85.7% and Isocitric dehydrogenases 91.7% and in *K. pneumoniae* it was 93%, 91%, 92% and 89% respectively (Table 4 and 5)

4. Discussion

Most of the studies on environmental toxicology dealing with microorganisms have been concerned with determination of a concentration of a metal or metal complex that is inhibitory and or lethal to the target organism, under artificial laboratory conditions (Babich and Stotzky, 1980; Bagde and Varma, 1982; Bhagwat and Srivastava, 1993). Inhibitory or lethal concentration of a metal is dependent on various factors. It is dependent both on metal and on the organism. The susceptibility of different species to heavy metals varies enormously (Bryan, 1971). Sensitivity and susceptibility of an organism to heavy metal attack varies considerably among different species and also between populations of one single strain depending on physiological state of the culture (Kaltwasser and Frings, 1980; Kruseger and Koloxziej, 1977).

Arsenic compounds have been widely used as biocides both for research purposes and in agriculture, industry, and medicine because of their toxicity to microorganisms, plants, insects, and mammals. They are also used as selective enzyme inhibitors in biochemical research. Several reports indicated that low concentration of metals is essential for the growth, but increasing concentration levels of it are inhibitory to growth and may even exert lethal effects on the organisms (Sugio et al., 1988; Angadi and Mathad, 1994).

In the present investigation it was observed that as the metal concentration in the medium increased, growth of the cells steadily decreased until the MIC concentration, where no growth was observed (Fig. 1 and Fig. 2). While in the control set immediate growth was observed, growth in cultures with lower concentrations other than MIC of heavy metal started late in cultures with inhibitor, a lag period of up to 6 to 12 hours was prominent. Similar results were reported by other researchers too (Komura and Izaki, 1971; Bagde and Varma, 1982). The lag in the presence of an inhibitor may be ceased by association of a high concentration of the inhibitor ion with membrane fraction resulting in a highly expanded membrane which is ineffective in transporting material needed for normal growth of the organism. Due to prolonged lag phase there is delay in log phase.

In the present study MIC of arsenic to *S. epidermidis* was found to be 200 ppm and to *K. pneumoniae* it was found to be 20 ppm. The same organisms when tested against silver by Surve and Bagde (2009) got very less MIC values as compared to this study (20 ppm for *S. epidermidis* and 3 ppm for *K. pneumoniae*). It was also reported that when these organisms were exposed to silver metal, there was decrease in protein, DNA and RNA contents (Surve and Bagde, 2009). Bagde and Varma (1982) reported a progressive decrease in protein, DNA, RNA content in *E. coli* and *Aerobacter aerogenes* against heavy metal lead. About 180 and 145 ppm of lead concentrations inhibited the growth, DNA and protein contents in *E. coli* and *A. aerogenes* respectively. In the present study too results of the protein, DNA, RNA contents of the cells of *S. epidermidis* and *K. pneumoniae* followed exactly the same pattern. Further it was also observed that as the growth decreased the content of protein, DNA and RNA of the cells decreased correspondingly.

Hwang Der-Ren et al. (2004) studied the effect of arsenic trioxide against Hepatitis C virus replication and showed that As_2O_3 inhibited HCV replication at sub-micromolar concentrations (0.35 μM). Arsenic was also found to be effective against *E. coli* by 0.5 mM concentration (Rossman et al., 1977). The study of arsenic against *E. coli* showed that arsenic inhibited one or more steps in the post-replication repair pathways of DNA. Guha and Mookerjee (1979) reported that when *E. coli* cells were exposed to nickel, Protein, RNA and DNA synthesis decreased. Similar results were reported in the present study also.

When heavy metal comes in contact with the cell, it is first taken up by the cell wall and then gets localized in

different parts of the cell. In the present study maximum metal got localized in cell membrane fraction which was in agreement to the observations made by other workers (Tornabe and Edwards, 1972 and Mitra et al., 1975). Mitra et al., (1975) however reported that the resistant or accommodated cells of *E. coli* contained maximum percentage of cadmium in cell wall rather than membrane. The reason for maximum percentage of arsenic being present in the membrane fractions of *S. epidermidis* and *K. pneumoniae* may be that the membrane might be the prime target for arsenic action. This can be substantiated by the fact that in bacteria the respiratory enzymes are located in the membrane. This is supported by our observations on inhibition of dehydrogenases of the TCA cycle. The four dehydrogenases involved in the TCA cycle, glutamic, succinic, α -ketoglutaric and isocitric dehydrogenases were inhibited to a greater extent, when bacterial cells were exposed to arsenic (Table 2). Due to this the supply of energy rich compounds like ATP got considerably reduced and thereby synthesis of macromolecules like protein, DNA and RNA declined and subsequently the growth of the cells got ceased (Wimpeny and Cole, 1968; Guha and Mookerjee, 1979; Martinez et al., 1991).

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Table 1. Inhibitory effect of Arsenic on the growth and macromolecular synthesis of *S. epidermidis*

Arsenic Concentration (ppm)	Growth inhibition %	Protein inhibition %	DNA inhibition %	RNA inhibition %
0	0	0	0	0
50	50	33	25	44
100	89	49	64	76
150	90.7	57	76	80
200	100	100	100	100

Table 2. Inhibitory effect of Arsenic on the growth and macromolecular synthesis of *K. pneumonia*

Arsenic Concentration (ppm)	Growth inhibition %	Protein inhibition %	DNA inhibition %	RNA inhibition %
0	0	0	0	0
5	38.3	22.1	51.1	48
10	91.2	65	78.3	88.2
15	95.6	73	86	90.3
20	100	100	100	100

Table 3. Distribution of Arsenic in sub cellular fractions of *S. epidermidis* and *K. pneumoniae* after 48 hours of treatment

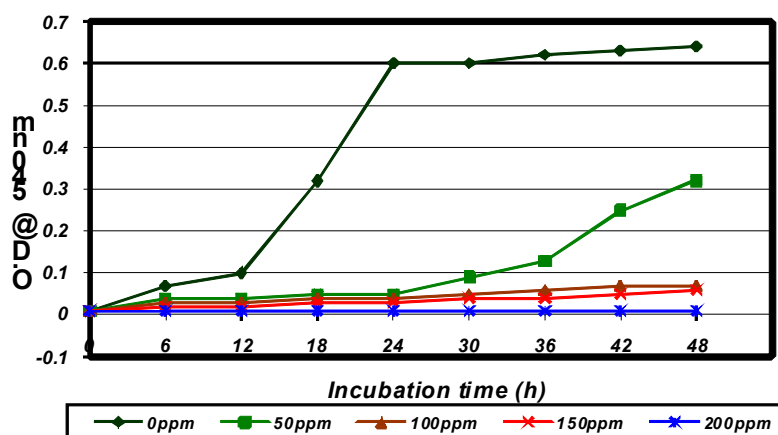
Fractions	<i>S. epidermidis</i>		<i>K. pneumoniae</i>	
	Standard Deviation	Percentage of Arsenic	Standard Deviation	Percentage of Arsenic
Cell wall	1.2	24.5	0.5	20
Cell membrane	0.8	32.5	1.1	35
Cytoplasm	1.1	43	2.8	45

Table 4. Effect of Arsenic on the activity of dehydrogenases of *S. epidermidis*

Enzymes	Control			Activity with Arsenic		
	O.D.	Activity %	Inhibition %	O.D.	Activity (%)	Inhibition (%)
Glutamic	0.15	100	0	0.01	6.6	93.4
Succinic	0.13	100	0	0.02	15.4	84.6
α Ketoglutaric	0.14	100	0	0.02	14.3	85.7
Isocitric	0.12	100	0	0.01	8.3	91.7

Table 5. Effect of Arsenic on the activity of dehydrogenases of *K. pneumoniae*

Dehydrogenases enzymes	Control			Activity with Arsenic		
	O.D.	Activity %	Inhibition %	O.D.	Activity (%)	Inhibition (%)
Glutamic	0.14	100	0	0.01	7.14	93
Succinic	0.11	100	0	0.01	9.09	91
α Ketoglutaric	0.12	100	0	0.01	8.33	92
Isocitric	0.09	100	0	0.01	11.11	89

Figure 1. Effect of various concentrations of Arsenic on growth of *S. epidermidis* at 540 nm

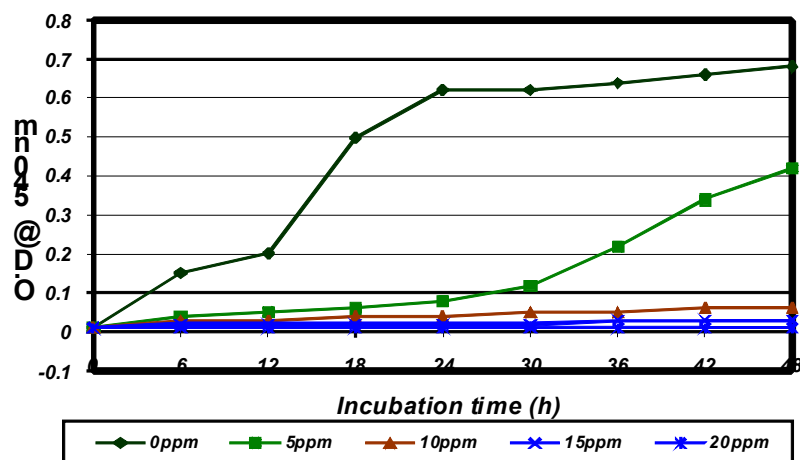


Figure 2. Effect of various concentrations of Arsenic on growth of *K. pneumoniae* at 540 nm

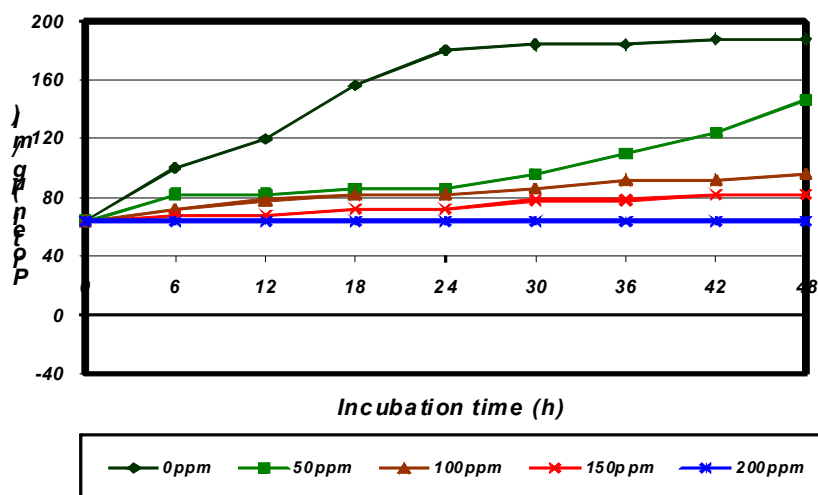


Figure 3. Effect of various concentrations of Arsenic on Protein contents of *S. epidermidis* in µg/ml of sample

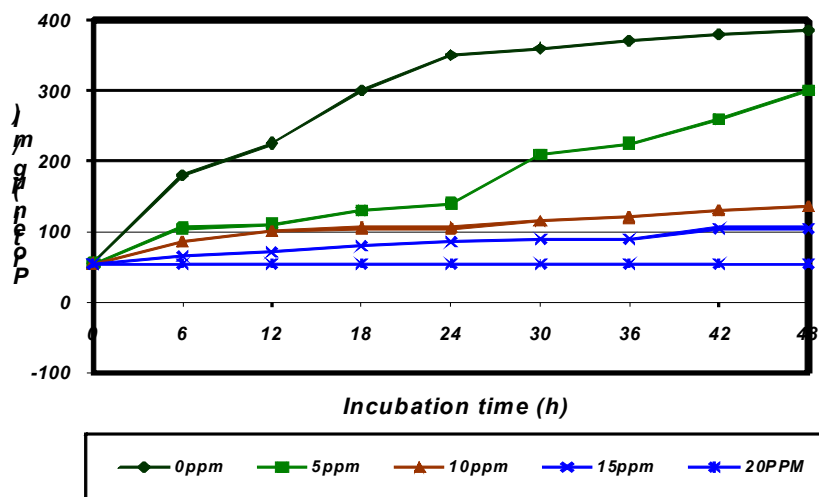


Figure 4. Effect of various concentrations of Arsenic on protein contents of *K. pneumoniae* in µg/ml of sample

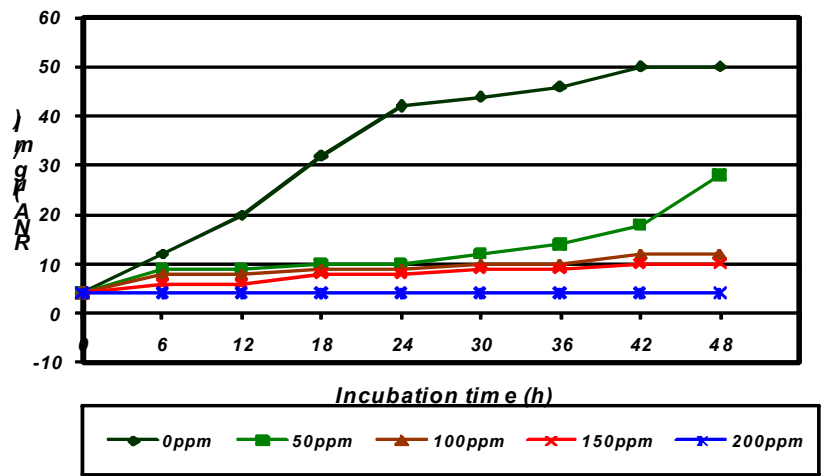


Figure 5. Effect of various concentrations of Arsenic on RNA contents of *S. epidermidis* in $\mu\text{g/ml}$ of sample

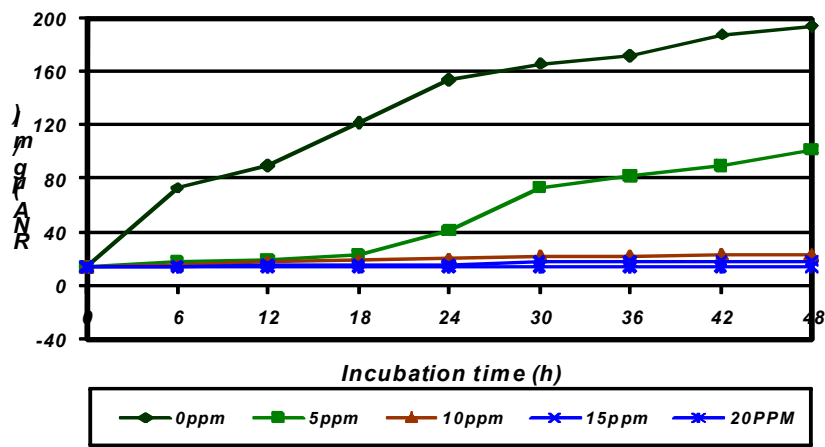


Figure 6. Effect of various concentrations of Arsenic on RNA contents of *K. pneumoniae* in $\mu\text{g/ml}$ of sample

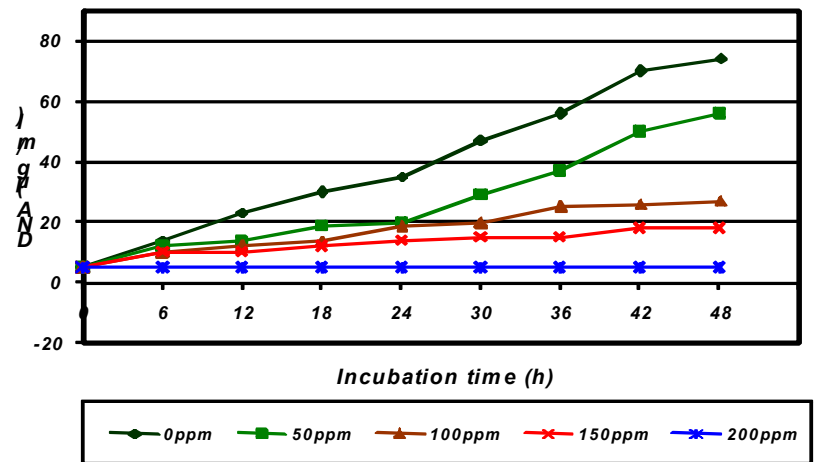


Figure 7. Effect of various concentrations of Arsenic on DNA contents of *S. epidermidis* in $\mu\text{g/ml}$ of sample

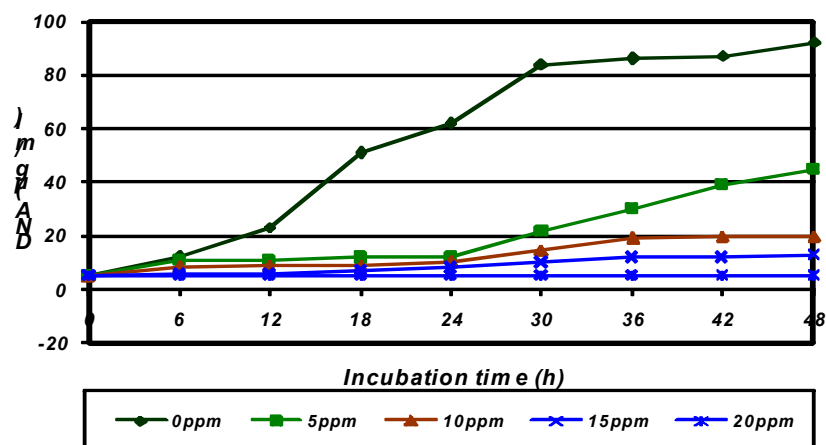


Figure 8. Effect of various concentrations of Arsenic on DNA contents of *K. pneumoniae* in µg/ml of sample