PLASMID REDUCTION VIA SODIUM ARSENITE IN ESCHERICHIA COLI

*Shiva Ahmadishoar1 and Saman Mahdavi2
1Department of Microbiology, Malekan Branch, Islamic Azad University, Malekan, Iran
2Department of Microbiology, Maraghe Branch, Islamic Azad University, Maraghe, Iran
*Author for Correspondence

ABSTRACT
The effects, at sub-MIC concentration (25mg/L), of sodium arsenite on different bacterial functions were studied. This compound reduced the killing activity of nalidixic acid, amikacin, and meropenem. It also promoted the loss of F’lac from bacterial hosts and increased the number of recombinants in conjugation and transduction experiments. Transposition of Tn9 was also enhanced by the salt. In addition, sodium arsenite abolished the lethal effect of the temperature against thermo-susceptible DNA synthesis mutants in a similar manner to that seen in an anaerobic environment. Finally at a low dose, it induced the SOS response, and the related production of the recA-dependent enzymes and plasmid were reduced as the sodium arsenite concentration was increased. It has been suggested that arsenite primarily affects the uvrA gene product influencing the other bacterial functions studied. The energetic depletion caused by this compound appears to play a role in the activity of autolytic enzymes.

Keywords: Sodium Arsenite, Plasmid Reduction, Antagonistic Activity against Drugs and Temperature, General Recombination, Transposition, SOS Induction

INTRODUCTION
Arsenic is widely spread in nature. Its presence in the environment is mainly related to volcanic activity but it is also released by other natural or industrial processes (Lewis, 2000; Caserola et al., 1982). Bacteria that are therefore exposed to arsenic from a variety of sources have acquired genetic information in order to resist the toxic effect of this metalloid and related compounds (Bentley and Chasteen, 2002; Butcher et al., 2000; Carlin et al., 1995; Debbia et al., 1995).

Arsenic toxicity depends on its oxidation state; in particular trivalent arsenicals are more toxic than pentavalent derivatives due to their high reactivity with the sulfhydryl group of proteins (Kondo et al., 2000). The great majority of information on the biological effects of these compounds was obtained from studies on mammalian cells and there is a universal agreement in considering this metalloid a serious carcinogen (Bates et al., 1992; Kondo et al., 2000). Arsenic, however, shows reduced toxicity against mammalian cells in comparison to that found on bacteria. Despite the extensive number of reports about the mechanisms used by microorganisms to escape the lethal activity of this metalloid, information about the role of arsenic on bacterial metabolism is scarce. In particular, an antagonistic effect between sodium arsenite and nalidixic acid has been reported against Klebsiella pneumoniae and E. coli strains (Debbia et al., 1995). However, the basis of this phenomenon has not yet been elucidated. It has been also observed that arsenite decreases the level of ATP and interferes with DNA synthesis and repair in bacterial cells (Dolcino et al., 2002). These processes depend in part or completely on a functional recA gene product and also appear to be mediated by the intracellular ATP concentration (Elionopoulos and Moellering, 1996). General recombination and transposition are also regulated by the above bacterial functions (Cervantes et al., 1994). But, the role of arsenic in these genetic events has never been evaluated. On the other hand, reduction of the ATP level in the cell causes an energetic depletion which could influence the activity of many enzymes (Wayne, 2002). Especially energy-dependent enzymes which promote cell death under restrictive growth conditions (Kreuzer and Cozzarelli, 1974; Quillardet and Hofnung, 1985). In this latter case exploring the effect of sodium arsenite on DNA metabolism in thermo-sensitive strains might be of some interest. It has been reported that concentrations up to 650 mg/L (5mM) of the sodium arsenite caused no appreciable loss of viability, while physiological perturbations were observed at 25 mg/L.
(Dolcino, 2002), a concentration at least ten-fold lower than the minimal inhibitory concentration (256-512 mg/L). This level is approximately 2 orders of magnitude below the dose generally used (0.25-0.5 x MIC) to study physiological disorders induced in bacteria by an antimicrobial agent (Craig and Kleckner, 1987). Thus it appears of some interest to explore the physiological perturbations induced by a sub-inhibitory concentration (25 mg/L) of sodium arsenite on several and apparently unrelated functions in bacteria.

This study was addressed to evaluate the role of sodium arsenite on the bactericidal activity of nalidixic acid, and other antibiotics characterized by a different mode of action such as amikacin and meropenem. The stability of Flac plasmid in strains cultured in the presence of the salt was also explored.

In addition, the effects of arsenite on other bacterial functions such as general recombination and transposition, the survival of thermo-sensitive mutants for dnaA46, gyrA43 and gyrB41 functions cultured at 43°C were studied. Finally the production of the SOS response was evaluated in bacteria exposed to different concentrations of sodium arsenite using a quantitative method.

MATERIALS AND METHODS
A. Bacterial Strains and Culture Conditions
Escherichia coli AB1157 and its various derivatives used in this study were described elsewhere. They were originally obtained from (Berlyn, 1998) of the E.coli Genetic Stock Center, with the exception of strain PQ37 which was a generous gift of Philippe Quillardet of the Institute Pasteur, Paris, France. Strains carrying relevant characteristics for this study were: CTR4610 (dnaA46[Ts]), KNK453 (gyrA43[Ts]), and N4177 (gyrB41[Ts]) All these mutants, cultured at43°C, resulted defective for DNA synthesis but they continued to divide and form non-nucleated cells because other cellular functions are not affected. HfrC was used as donor strain in mating experiments. The LB rich medium and the minimal medium were those described by Miller. Media were supplemented with thymine (25 mg/L) when necessary (Caserola, 1982; Louarn et al., 1984; Miller, 1972).

B. Chemicals
The antimicrobials (nalidixic acid, amikacin, imipenem, and mitomycin C) as well as sodium arsenite were obtained from Sigma Chemical (Milan, Italy). Sterile stock solutions of the compounds were prepared from the standard reference powder in accordance with the instructions of the respective manufacturer and when necessary were protected from light throughout all manipulations.

C. Susceptibility Tests
Minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined following the microdilution procedure suggested by the National Committee for Clinical Laboratory Standards (Mukhopadhayay, 2002). Using Mueller-Hinton (Difco Laboratories, Milan, Italy) as test medium. Time-kill experiments were performed by adding the drug at concentrations corresponding to 4 x MIC, to log-phase bacterial cultures diluted to 106-107 CFU/ml growing in 500 ml flasks at 37°C. Just before the compounds were added (zero time) and at 2, 4 and 6h thereafter, bacterial counts were carried out by determining CFU on agar plates. Colonies were counted after 48h of incubation at 35°C. The anaerobic environment was established by culturing the test bacteria for 24 h in broth covered with 2 cm of liquid paraffin. The broth used, and the flasks employed for anaerobic experiments were maintained in an anaerobic glove box (model 1024; Forma Scientific, Marietta Ohio) for 24 h. The standard inoculum of bacteria was dispersed in the containers and then they were covered with 2 cm of liquid paraffin and transferred to an aerobic environment. Bacterial samples were collected using a Hamilton syringe. When thermo-sensitive mutants were studied bacterial cultures incubated at 32°C were adjusted to the usual cell density and divided in equal volumes. Two samples were incubated at 43°C for 10 min and then sodium arsenite at 25 mg/L was added to one test tube, the others were treated in similar manner and maintained at 32°C. Bacterial counts were determined as described above.

D. Methods for Plasmid Elimination
Bacteria were grown overnight in Mueller-Hinton Broth (MHB) containing kanamycin (25 mg/L) to ensure Flac: Tn5 presence. Under these conditions the percentage of plasmid-free cells was less than
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0.1%. After the drug was removed, by washing the cells in saline buffer, approximately 5 x 103 bacteria were cultured in MHB with 25 mg/L of sodium arsenite. A tube of arsenite-free MHB was used as a control. After incubation for 18-20 h at 37°C the suspensions were diluted 100-fold in pre-warmed drug-free medium and incubated for a further 90 min. Controls were diluted similarly. Then bacteria were diluted in saline buffer and plated on drug-free Mueller-Hinton agar (MHA). After incubation for 18-20 h colonies were replicated onto kanamycin-free and antibiotic-containing MHA using velvets. Growth on antibiotic-containing media indicated the presence of the plasmid encoding that resistance. In some cases the presence of F’lac was detected by scoring the lactose-positive colonies on McConkey agar plates. The number of colonies on drug-containing plates was expressed as a fraction of the number of on drug-free plates (Craig and Kleckner, 1987).

E. Bacterial Conjugation and Transduction

General mating conditions for evaluating genetic exchange were previously described (Datta, 1983). Log phase cultures (2x108 CFU/ml) of donor (1 ml) and recipient (1 ml) strains cultured in LB medium, were mixed and incubated at the appropriate temperature for 60 min then washed by centrifugation and plated on selective minimal media. Thr-leu+ recombinants were counted after 48h incubation at 37°C. Control strains were also plated. The T4GT7 transduction was carried out by infecting (m.o.i.=1) log phase cultures (2x108 CFU/ml) of the recipient in the presence of tryptophan (25 mg/L). After 30 min at 37°C bacteria were washed by centrifugation and plated on selective minimal media. Thr-leu+ recombinants were counted after 48 h incubation at 37°C. To evaluate the role of sodium arsenite in the above genetic experiments, the tests were carried out simultaneously in a rich medium containing 25 mg/L of the compound.

F. Transposition

Log-phase bacteria suspended in LB broth containing 0.005M CaCl2 were infected with P1vir::Tn9 or P1Cmcr100. After 1 h at 32°C the microorganisms were plated on LB agar plates containing Cm 20 mg/L and EGTA 0.0025M. The effect of sodium arsenite upon transposition was tested by adding the salt during the infecting phase. Plates were then incubated at 37°C for 24-36 h (Craig and Kleckner, 1987).

G. Dosage of SOS Response

The procedure suggested by (Quillardet and Hofnung, 1985) was used.

RESULTS AND DISCUSSION

A. Antagonistic Effect of Sodium Arsenite on the Killing Rate of Various Drugs

In preliminary experiments, the susceptibility of AB1157 to nalidixic acid, amikacin, and meropenem was assessed by the microdilution broth method in the presence of 25 mg/L of sodium arsenite. The results obtained did not show any difference in the MIC values between the tests carried out with and without the arsenical salt. Thus the experiments were repeated using a dynamic bactericidal quantitative system. A similar behaviour was observed with amikacin and meropenem. The growth curve of the strain treated with the arsenic salt at 25 mg/L did not significantly differ from that of the control. A generation time of about 51 min was, in fact, detected for the control in comparison with 56 min noted in the strain exposed to sodium arsenite. When the same experiments were repeated employing each drug in combination with sodium arsenite a reduction of the killing rate was noted. In particular, at the end of the experiment the number of viable bacteria treated with nalidixic acid in combination with sodium arsenite was similar to that registered at the beginning of the test. While, under the same experimental conditions, a reduction of about 90% of the original number of bacteria, was observed when amikacin and meropenem were combined with arsenite.

B. Effect of Sodium Arsenite on the Stability of F’lac Plasmid

A microscopic examination of the bacterial cultures exposed to 25mg/L (0.05xMIC) of sodium arsenite revealed the presence of elongated forms, a condition known to promote plasmid loss (Craig and Kleckner, 1987).

F’lac stability was then evaluated after incubation of the host for 18-24 h at 37°C in the presence of sodium arsenite and nalidixic acid used as positive control. Cured cells (range: <0.1-37.2%, mean
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16.5%) were detected at a concentration of the salt which was about 20-fold lower than the MIC found, under these experimental conditions, in AB1157 (512 mg/L) (Table 1), while, as expected, nalidixic acid promoted Flac elimination (range: 13.9-58.4%, mean 36.2%) at a level corresponding to 0.5xMIC.

Table 1: Effect of sodium arsenite and nalidixic acid on the stability of F’lac in E.coli AB1157

<table>
<thead>
<tr>
<th>No. Recombinants/10^7 recipients with (in mg/L)</th>
<th>Sodium arsenite (25)+</th>
<th>Nalidixic acid (20)</th>
<th>Sodium arsenite (25)</th>
<th>None</th>
<th>Dosor</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.54±1.97 b</td>
<td>1.94±0.95 b</td>
<td>15.53±1.98 b</td>
<td>8.56±1.27 b</td>
<td>HfrC</td>
<td>Conjugation</td>
<td></td>
</tr>
<tr>
<td>3.49±1.08 b</td>
<td>2.01±0.25 b</td>
<td>6.99±0.84 b</td>
<td>3.76±0.82 b</td>
<td>T4GT7 (HfrC)</td>
<td>Transduction</td>
<td></td>
</tr>
<tr>
<td>271.6±84.145 b</td>
<td>74.8±22.45 b</td>
<td>1339±291.73 b</td>
<td>235.6±70.46 b</td>
<td>P1·Tn9</td>
<td>Transposition</td>
<td></td>
</tr>
</tbody>
</table>

of recombinants was comparable to that of the control. A similar phenomenon was observed in transduction experiments carried out employing the T4GT7 phage. The recovery of transductants, in fact, was enhanced by sodium arsenite by the same order of magnitude as that detected in the mating experiments, while as expected the number of recombinants was reduced by nalidixic acid (p ≤ 0.0028). When sodium arsenite was combined with nalidixic acid the arsenical salt appeared to abolish the effect of the quinolone. In fact, the number of transductants found were approximately the same as that registered in the control experiments (p = 0.1). As reported in Table 2 the presence of sodium arsenite, during the period of time that Tn9 transfers itself from phage P1 to bacterial genome, increased the frequency of chloramphenicol resistant isolates by about ten times (p ≤ 0.0028) in comparison with that observed in the control. Nalidixic acid reduced the number of transposed cells by about 90% (p ≤ 0.0028). The combination of arsenite with nalidixic acid was beneficial for the number of transposed bacteria, in that the number of colonies found on the plates was about the same as the control.

Table 2: Effect of sodium arsenite on conjugation, transduction and transposition

<table>
<thead>
<tr>
<th>Cured cells (Mean) *</th>
<th>Dosed used (mg/L)</th>
<th>MIC (mg/L)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.9-38.4 (36.18)</td>
<td>2</td>
<td>4</td>
<td>Nalidixic acid</td>
</tr>
<tr>
<td>10.1-37.2 (16.48)</td>
<td>2</td>
<td>512</td>
<td>Sodium arsenite</td>
</tr>
<tr>
<td>10.0-1.5 (0.88)</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

*a range of at least five separate experiments, b Recipient AB1157, c d p ≤ 0.0028 by paired Student’s test, p = 0.087 by paired Student’ test, p = 0.10 by paired Student’s test, f p = 0.015 by paired Student’s test

C. Role of Sodium Arsenite in General Recombination and Transposition

The influence of sodium arsenite in general recombination was evaluated in both conjugation and transduction experiments. In order to minimise the growth of revertants, thr-leu+ recombinants were selected. When bacterial conjugation between a HfrC donor strain and the recipient AB1157 was carried out in the presence of sodium arsenite the number of recombinants increased about 2 fold (p ≤ 0.0028) in comparison with that obtained in the control experiment (Table 2). Nalidixic acid, included as negative control, strongly reduced the number of recombinants. When sodium arsenite was combined with

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naldixic acid the number D. Effect of sodium arsenite on the growth rate of gyrA43, gyrB41 and dnaA46 thermo-sensitive mutants cultured at the non permissive temperature.

As reported sodium arsenite reduced the lethal effect of the temperature on gyrA43, gyrB41 and dnaA46 thermo-sensitive mutants cultured under non permissive conditions. In particular with the gyrB41 and dnaA46(Ts) strains, the addition of arsenite to the broth culture produced a bacteriostatic effect and at the end of the experiment the number of bacteria was similar to that of the original inoculum, while under the same experimental conditions gyrA43(Ts) resulted susceptible to the lethal effect of the temperature during the first 4 h of incubation at 43°C. After this period a bacteriostatic effect was noted. At the end of the experiment the number of survivors found in the presence of sodium arsenite was about two Log greater than that registered in non treated culture.

E. Growth of gyrA43, gyrB41 and dnaA46 Ts Mutants Cultured at the non Permissive Temperature under Anaerobic Environment

The anaerobic environment reduced the lethal effect of the temperature on gyrA43, gyrB41 and dnaA46 thermo-sensitive mutants cultured under non permissive situations. In particular with all the strains the growth conditions produced a bacteriostatic effect and at the end of the experiments the number of bacteria was similar to that of the original inoculum.

F. Quantitative Evaluation of the SOS Induction by Sodium Arsenite

As reported in Table 3 the arsenical salt caused a dose-dependent effect on SOS induction, in particular, at low concentrations (4 and 25 mg/L) there was an induction of the SOS response corresponding to 1.4 fold in comparison to that evaluated as basal level (p ≤ 0.00027). This was about one third with respect to that registered with mitomycin C employed as positive control. When the concentration of sodium arsenite was increased to 50 mg/L there was a reduction in the level of expression corresponding to 91% of the basal level (p = 0.011). At a concentration of 100 mg/L of sodium arsenite the level of expression was about 50% less than that detected in non-treated bacteria (p =0.017).

Table 3: Effect of sodium arsenite on recA expression

<table>
<thead>
<tr>
<th>Relative variation (fold)</th>
<th>Level of expression Mean ± SD</th>
<th>Dose (mg/L)</th>
<th>Inducing treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>309.08 ± 67.95</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>1.4^b</td>
<td>442.8 ± 82.205</td>
<td>4</td>
<td>Sodium arsenite</td>
</tr>
<tr>
<td>1.4^b</td>
<td>453.4 ± 93.407</td>
<td>25</td>
<td>Sodium arsenite</td>
</tr>
<tr>
<td>0.91^c</td>
<td>250.4 ± 41.253</td>
<td>50</td>
<td>Sodium arsenite</td>
</tr>
<tr>
<td>0.43^d</td>
<td>156 ± 21.909</td>
<td>100</td>
<td>Sodium arsenite</td>
</tr>
<tr>
<td>4.4^b</td>
<td>1028.8 ± 197.045</td>
<td>0.2</td>
<td>Mitomycin C</td>
</tr>
</tbody>
</table>

^a range of at least five separate experiments

^b p≤ 0.00027 by paired Student’s test

^c p = 0.011 by paired Student’s test

^d p = 0.017 by paired Student’s test

Discussion

Owing to its abundance in the environment and its toxicity for animal and vegetable tissues as well as for microorganisms, arsenic has been extensively studied either as a carcinogen agent or in order to understand the systems developed by living organisms to escape its toxic effect (Bates et al., 1972; Bentley and Chasteen, 2002; Caserola et al., 1982; Kondo et al., 2005). The present report suggests that sodium arsenite may induce a great variety of biological effects that have never been described before. In particular, it antagonised with the killing activity of naldixic acid, amikacin, and meropenem. It also promoted the loss of F’lac from bacterial hosts; it increased the number of recombinants in conjugation.
and transduction experiments, and the frequency of Tn9 transposition. In addition, it abolished the lethal effect of the temperature on thermo-susceptible DNA synthesis mutants in a similar manner to that observed under anaerobic conditions. Finally, at a low dose it induced the SOS response, conversely when the concentration of sodium arsenite was increased the production of these recA-dependent enzymes was reduced. In an attempt to explain all the above biological events some considerations and hypotheses can be argued. Under the experimental conditions employed here the only phenotypical alterations showed by the microorganisms exposed to arsenic was a little variation in their growth rate together with a slight induction of the autolytic enzymes. All these effects might act synergistically causing an altered physiological condition influencing the fitness of bacteria that do respond correctly to any biological stimulus. The reduced killing effect exhibited by sodium arsenite against the bactericidal activity of nalidixic acid, amikacin, and meropenem might be an example of this condition.

It is possible that among all the physiological perturbations caused by arsenic, some proteins or enzymes are in part or completely impaired causing a bacteriostatic event. Therefore, from a certain point of view sodium arsenite might be considered a sort of protein inhibitor, and since the association of this latter class of drug with all other antibiotics leads to a bacteriostatic effect (Diorio et al., 2005) this might explain the antagonistic activity between arsenic and the other drugs observed in this study irrespectively of their mode of action. Another possibility is that arsenic blocks the induction of the autolytic enzymes that the above antibiotics are known to trigger in this microorganism (Kreuzer and Cozzarelli, 1974; Nimmo, 1987; Quillardet and Hofnung, 1985).

Arsenite promotes F’lac loss in the host and this is probably due to the formation of filamentous cells (Craig and Kleckner, 1987). Due to the modest induction of the SOS response by the salt, as observed here.

The genetic events observed here are difficult to understand if the direct involvement of the recA gene product or the influence of sodium arsenite in DNA synthesis and repair are to be taken into consideration. Experiments carried out in this laboratory indicate that F’lac transfer is not affected by sodium arsenite at the concentration used here (25 mg/L), and takes place in non growing bacteria (Datta et al., 1983). And unpublished observations) supporting the idea that neither the mating pairs formation of the bacterial cells nor the conjugational DNA transfer are the targets of this metalloid. An intriguing hypothesis considers the role of sodium arsenite in the inhibition of the excision repair process mediated by the uvrA gene product. It has been reported that in strains defective for this latter function there is an increase of recombinants in transduction and conjugation, as well as in transposition (Eliopoulos and Moellering, 1996). Thus the high number of genetic reactions registered in this study could be ascribed to inhibition of the excision repair gene product by sodium arsenite.

Several factors are known to phenol typically suppress dnaA46(Ts) mutations (Hanada et al., 2000; Hirota et al., 1968; Kreuzer and Cozzarelli, 1974). While no data are available for similar phenomena in gyrA43(Ts) and gyrB41(Ts). The present findings show evidence of a bacteriostatic effect induced by sodium arsenite on the thermo-sensitive mutants studied. Similar results were obtained by culturing the mutants under anaerobic conditions. This latter point supports the hypothesis that an energetic depletion might be responsible for the survival of these strains under non permissive conditions.

As mentioned above, arsenic affects ATP level in bacteria by inhibiting the ketoglutarate dehydrogenase complex (Craig and Kleckner, 1987). This also occurs under anaerobic conditions where the greatmajority of aerobes modify their Krebs cycle by reducing ATP production (Nimmo, 1987). Since autolytic proteins need energy to carry out their biological functions (Miller, 1972; Quillardet and Hofnung, 1985; Leonard, 1980). Either the presence of arsenic or the anaerobic environment could reduce the necessary amount of ATP so preventing specific enzymes from digesting the bacterial cell wall. At a...
concentration of sodium arsenite lower than that used here (<25 mg/L) there was a moderate induction of the SOS response.

This phenomenon appears difficult to explain in terms of arsenite mutagenicity, a condition known to activate the DNA repair mechanism, because there is no evidence that the salt exhibits this property. If, however, the uvrA gene product is impaired by arsenic, DNA is not promptly repaired, bacteria are stimulated to induce the SOS response. This observation is also in agreement with that reported by (Caserola et al., 1982), who found that the basal level of SOS expression in the uvrA defective mutant was about 1.4 fold greater than that of the wild-type counterpart.

In conclusion sodium arsenite induces pleiotropic effects in bacteria that may be attributed to its interaction with proteins. This phenomenon affects, with different degrees of susceptibility, various bacterial reactions. In particular the uvrA gene product appears to be one of the more susceptible targets. Microorganisms impaired in this function tend to produce a higher level of SOS response than in basal conditions. This promotes an increase in DNA recombination processes, and leads to the formation of a certain number of filamentous forms with concomitant disorders in the division of the bacterial cell. The depletion in ATP production, confirmed by the experiments carried out under anaerobic environment seems to be another phenomenon related to the mode of action of sodium arsenite. Finally, sodium arsenite appears to be an interesting compound exhibiting a dose-dependent pleiotropic effect which could be used to study apparently unrelated bacterial responses.

REFERENCES
Butcher BG, Deane SM and Rawlings DE (2000). The chromosomal arsenic resistance genes of Thiobacillus ferrooxidans have an unusual arrangement and confer increased arsenic an antimony resistance to Escherichia coli. Applied and Environmental Microbiology.
Research Article


Wayne PA (2002). National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Twelfth Informational Supplement: M100-S12 22(1) NCCLS.
