"The reduction of copper in *Streptococcus pneumoniae* by thioredoxin and copper chaperone CupA"

By

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STATEMENT BY AUTHOR

The Internship Report titled **"The reduction of copper in Streptococcus pneumoniae by thioredoxin and copper chaperone CupA"** and **"Optimization of antibiotic assay using Smarticle based method"** prepared by Preetham Hosur has been submitted in partial fulfillment of requirements for a master's degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Michael D. L. Johnson, PhD
Assistant Professor, Immunobiology

Defense date
4/27/18

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1 Background

Streptococcus pneumoniae (S. pneumoniae) is a gram-positive pathogen and leading cause of serious and life threating bacterial infection including pneumonia, bacteremia and meningitis. Worldwide, annually 1.1 million of children die from the pneumococcal infections and most of the children are under the age of (Tong N, 2013). For *S. pneumoniae* to survive and cause disease in the diverse environment related with these infections, it must have a capacity to sense and adjust the various environmental conditions, which also includes the changes in the concentration of metal ions in the host tissues.

Metals serve as vital nutrients to all biological systems and it has been estimated almost nearly 50% of all enzymes in the cells require a metal cofactor (Monosson, 2012). During infections, bacteria acquire all metals necessary for survival from within the host, such as iron, calcium, and manganese, but must also efflux metals that are toxic or in excess such as copper (Casey AL1, 2010 Jan 7). Despite of the critical role of copper in a wide range of mammalian biological processes, copper has been used as an antimicrobial agent for thousands of years and currently, is used in healthcare and agriculture (Marie I. Samanovic, Copper in microbial pathogenesis: meddling with the metal, Feb 6, 2012).

To avoid the excess copper toxicity, bacteria have developed mechanisms to protect themselves from the toxic effects of the copper ions. The principle bacterial and fungal mechanism to overcome copper toxicity is to have a transmembrane copper export occurring from the cytoplasm into the periplasmic space or into the extracellular space (Gregor Grass, 2011).

To counter this copper toxicity, bacteria have developed highly effective copper efflux system to remove the excess cytosolic copper which plays a vital role during the infection (Erin S. Honsa, 2013 Dec 4) (Michael D. L. Johnson T. E.-F., 2015). *S. pneumoniae* contains significant cell

associated copper where adaptation to high cuprous environment in the cell may be imperative to the invasive disease (Holden, 21 December 2001) . During infection, both the influx and efflux systems of manganese and zinc, calcium efflux and copper efflux plays a major role in *pneumococcus* system (Erin S. Honsa, 2013 Dec 4) (Holden, 21 December 2001). Also, during infection, the host is thought to sequester the vital metals iron, manganese and zinc, termed nutritional immunity (Hood MI, 2012). Concurrent with the sequestration of vital metals, the host also exploits the toxic effects of metals, consisting of copper to fight against invading pathogens (Michael D. L. Johnson T. E.-F., 2015) (Yue Fu F.-M. J., 2014) (James E. Cassat, 2013 May 15).

Copper in the biological systems can exist either in the cuprous Cu (I) state or in the oxidized cupric Cu (II) state. Copper can lead to the generation of reactive oxygen species (ROS) i.e. free radicals via Fenton reactions. In this reaction, the cuprous is oxidized in the presence of hydrogen peroxide leading to the formation of cupric Cu(II) and free radicals **($Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH\cdot + OH^{-}$)** (G.McEwan, 2017).

From the previous research, it was shown that the streptococcal species need a functional CopA and it was found that a novel *S. pneumoniae* system of copper toxicity is mismetallating a ribonucleotide reductase (NrdF), by displacing manganese, thus inhibiting the nucleotide synthesis (Michael D. L. Johnson T. E.-F., 2015). This in turn leads to the decreased replication and increased transcription of anaerobic nucleotide synthesis pathway **(**Figure 1**)** (Michael D. L. Johnson T. E.-F., 2015).

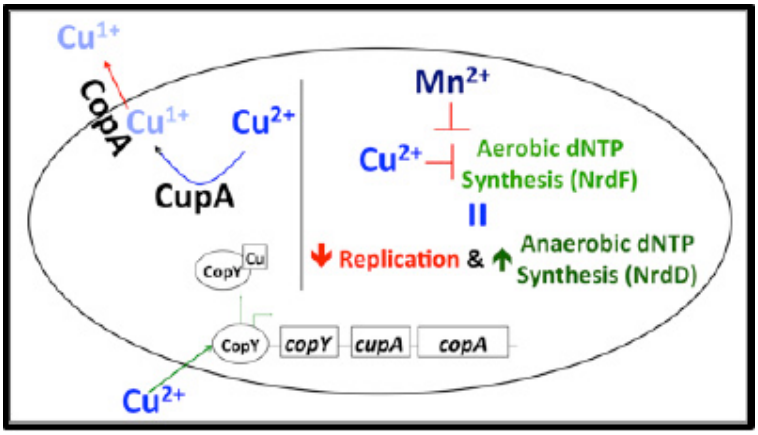


Figure 1 Schematic representation of the primary toxic mechanism of Copper in *S. pneumoniae* (Michael D. L. Johnson T. E.-F., 2015)

To address copper stress, *S. pneumoniae* use different pathways such as the *cop* operon (Figure 2**)**. This pathway has been demonstrated to be essential for copper resistance in *S. pneumoniae* (Yue Fu F.-M. J., 2014). This operon pathway is mediated by different essential elements; CopY, a copper dependent repressor of the *cop* operon, CupA a copper chaperon protein that can reduce copper, and CopA, P_{1B} type ATPase that exports copper from the bacteria (Lutsenko, 2010) (Sulman Shafeeq, 2011) (Miranda J. Neubert, 2017 Sep-Oct).

From the copper export system, Cu^{2+} enters into the bacterial system and it is exported as Cu^{1+}. Here we trying to study what is the mechanism that is helping the CupA chaperon in reducing the copper from Cu^{2+} to Cu^{1+}.

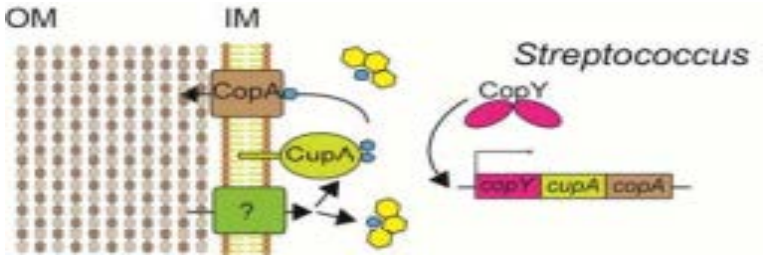


Figure 2 *cop* operon pathway (Yue Fu F.-M. J., 2014).

2 Rationale

The hypothesis of the study was to investigate the reduction of copper in *S. pneumoniae* with the help of the copper chaperon and thioredoxin. Through the transcriptional profiling with DNA microarrays, we found that several different genes were up- and downregulated under copper stress. It was observed from the microarray data that thioredoxin genes were upregulated under copper stress. Thioredoxin acts as a reductant in many cellular reactions, such as ribonucleotide reactions, transfer the reducing equivalents from cytoplasm to cell via membrane bound oxidoreductases (Samuel Lee, 2013 Apr 1). It was also seen, using a Cu^{1+} reporter that CupA, the *cop* operon copper chaperone, is able to reduce Cu^{2+} to Cu^{1+} (Figure 3). Therefore, our hypothesis was to elucidate if thioredoxin and thioredoxin reductase with CupA would have a synergistic effect to reduce the copper.

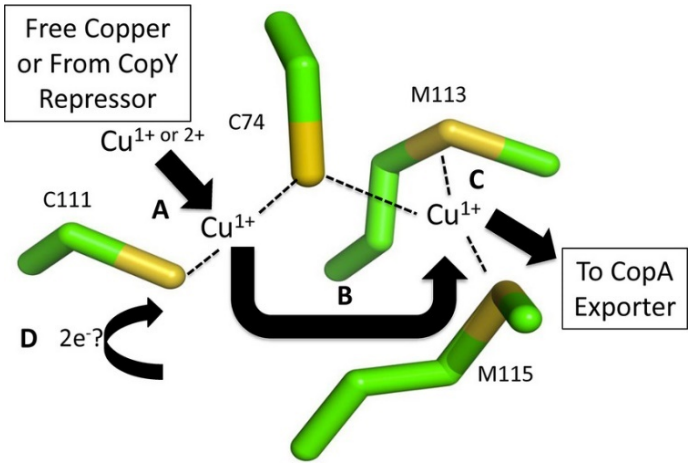


Figure 3 Cupredoxin mechanism of CupA (Miranda J. Neubert, 2017 Sep-Oct).

3 Methods Materials

3.1 Protein Homology and domain prediction

The protein sequences were entered or the FASTA file was selected into the NIH Protein Basic Local Alignment Search Tool (BLASTp) (J.Lipman, October 1990). The output of the BLAST predicted the protein domain and aligned with the similar sequences.

3.2 Protein Production

3.2.1 Expression of CopY

The CupA gene was present on an ampicillin resistant plasmid and transformed into BL21 gold cells. A single amp^r colony was used to inoculate a 100-ml flask of Luria broth (LB) containing ampicillin (100 mg/ml), and the cells in the culture were grown overnight. Cell were collected by centrifugation at 3,000 g, and the supernatant was discarded. The resultant pellet of cells was used to inoculate a 2.8-liter shaker flask of Terrific broth containing + 4% glycerol. Cells were grown in Terrific broth at 37°C until the optical density at 600nm ($O.D._{600}$) of the culture was 0.6-0.8. The temperature was lowered to 17°C for overnight growth, and expression of CupA was induced by adding 1M isopropyl- D-thiogalactopyranoside (IPTG). After overnight growth, cells were harvested by centrifugation at 6,000 g for 20 min at 4°C. The supernatant was discarded, and cell pellets were stored at -80°C.

3.3.2 Purification of His Tagged Proteins (CupA, TrxA and TrxB)

Cell pellets were resuspended in buffer A (preferably cold) consisting of 50 mM Tris pH 7.4, 200 mM NaCl, 25 mM Imidazole, 5% glycerol, 1 mM NaN_3, the protease inhibitor tablet which helps to inhibit protein degradation, DNAse which helps to breakdown the DNA which compete with

CopY during purification and 1 mM PMSF it inactivates serine and some cysteine proteases. Then the cells are lysed via sonicator to realize the proteins within their cytosol. Cells are centrifuged at for 20 min at 15000 x g to separate the insoluble fraction from the supernatant, which contained the desired protein. Then the supernatant was filtered and collected in 50ml conical flask. Using the AKTA Fast Protein Liquid Chromatography (FPLC), the supernatant (lysate) was then passed over a gel containing Ni^{2+}, which binds to negatively charged moieties, especially poly-histidine tags. Nonspecific binding proteins were washed away with buffer. The desired protein was eluted from the Ni^{2+} column using a buffer B which has high concentration of imidazole (the side chain unit of histidine), which outcompetes the protein for the nickel resin, thus eluting the protein off the nickel column. Then this concentrated protein was purified over a Superdex S200 16/200 column using an AKTA pure FPLC (GE Healthcare). Fractions containing desired protein were pooled and concentrated for immediate use or frozen it using liquid nitrogen and stored at -80°C.

3.3 Ligation Independent Cloning Protocol (LIC)

3.3.1 Preparation of Vector DNA

Vector pMCSG7 140-157 (The Biodesign Institute/Arizona State University, n.d.) is designed with the use SSp1 to linearize the vector (restriction site AATATT) with vector DNA-36 µl, 10x Buffer 2 (NEB2)-4 µl, SSp1-2µl (5 U/µl) and ddH_2O-8 µl, bring up to 50 µl total volume. Restriction digest is performed for at least 2 hours at 37ºC, followed by heat inactivation at 65ºC for 20 minutes.

Run a sample of the vector on an agarose gel. Once the band is seen for uncut vector, then all the sample must be gel purified to limit false positives. PCR purification is carried out for the vector DNA form the gel pieces. Elute the digested vector DNA in 50 µl ddH_2O in a 1.5-ml microfuge

tube once the vector is obtained, LIC treatment of the vector is done with PCR reaction which includes: Linearized Vector-50 µl, NEB Buffer 2-10 µl, 1 M DTT-0.5 µl, 100 mM GTP-2.5 µl, T4 DNA Polymerase-2 µl and ddH_2O-35 µl. Then run on thermocycler at 22^oC for 30 min, 75^oC for 20 min and at 4^oC to hold. Sample is taken from thermocycler and PCR purified the sample by eluting it in ddH_2O, aiming for a final concentration of 30 ng/µl.

3.3.2 Preparation of the insert for TrxA and TrxB

To create an insert with complementary overhangs to the LIC vectors that are made the following primers must be used:

Forward primer 5'-TACTTCCAATCCAATGCGXX…-3'

Reverse primer 5'-TTATCCACTTCCAATGCGCTAXX…-3'

Perform a PCR to clone out TrxA and TrxB of host plasmid using the above overhang sequences to create LIC compatible primers. PCR purification was carried out Eluting in 30 µl of ddH_2O. Phosphorylation of the PCR product is done by carrying out the reaction which includes: PCR Product-30 µl, 10x T4 ligase buffer-4 µl, T4 PNK-1 µl, and ddH2O-5 µl (Total volume 40 µl) .Then run on thermocycler protocol LICOVER at 37^oC for 90 min, 65^oC for 20 min and at 4^oC to hold. Creating LIC overhangs by adding to previous reaction: NEB Buffer 2-2.7 µl, 1M DTT-0.5 µl, dCTP-1.25 µl, T4 DNA Polymerase-0.5 µl, ddH_2O-5.05 µl (Total volume 50 µl). PCR clean the sample, eluting in 40 µl ddH_2O. Anneal vector and insert by adding 30 ng of insert to 30 ng of vector in an Eppendorf tube, incubate at room temperature for 10-20 minutes. Transform into DH5α cells using 2 µl of sample and standard DH5α protocol.

3.3.3 Preparation of plasmid mini-preps

Single colonies were picked from the positive plate and inoculate into the above tubes. Incubate overnight at 37°C in a shaker/incubator. Spin for 10 min at 4,000 rpm (table top centrifuge) and discard the supernatant. Then resuspended the pellets in the appropriate buffer to prepare plasmid mini-preps. Take the concentration of the DNA and send it for DNA sequencing.

3.4 Cupredoxin assay

For V_{max} and K_m studies, bathocuproinedisulfonic acid (BCS) was added at a 100mM concentration, 50 µM CupA (or mutations thereof) with varying amounts of $CuSO_4$ (5 µM to 625 µM) in TBS (chelating CopY buffer) to a final volume of 200 µl to a cuvette. The absorbance at 490 nm was read in a spectrophotometer, with TBS with $CuSO_4$ and BC used as the blank. For B_{max} and T_{50} in the timed measurements, TBS, 100 mM BCS, 50 CupA (or mutations thereof), 50 µM $CuSO_4$ (in that order) were added to a cuvette for a final volume of 1 mL and tracked over time, with TBS with $CuSO_4$ and BCS used as the blank. The Cu solution of varying concentration is prepared for the injection column. Thioredoxin (TrxA) and Thioredoxin Reductase (TrxB) of varying concentration was added to make up the final volume 180 µl.

Biotek Cytation 5 multimode plate reader setup

For the assay read out we use the protocol, set on the Biotek Cytation 5 multimode plate reader for regular use which includes the different steps like (Figure 4)

Dispense

Start of kinetics

a) Shake

b) Read

End of kinetics

These steps were used once for a single well sample if multiple, repeat the steps for required number of wells.

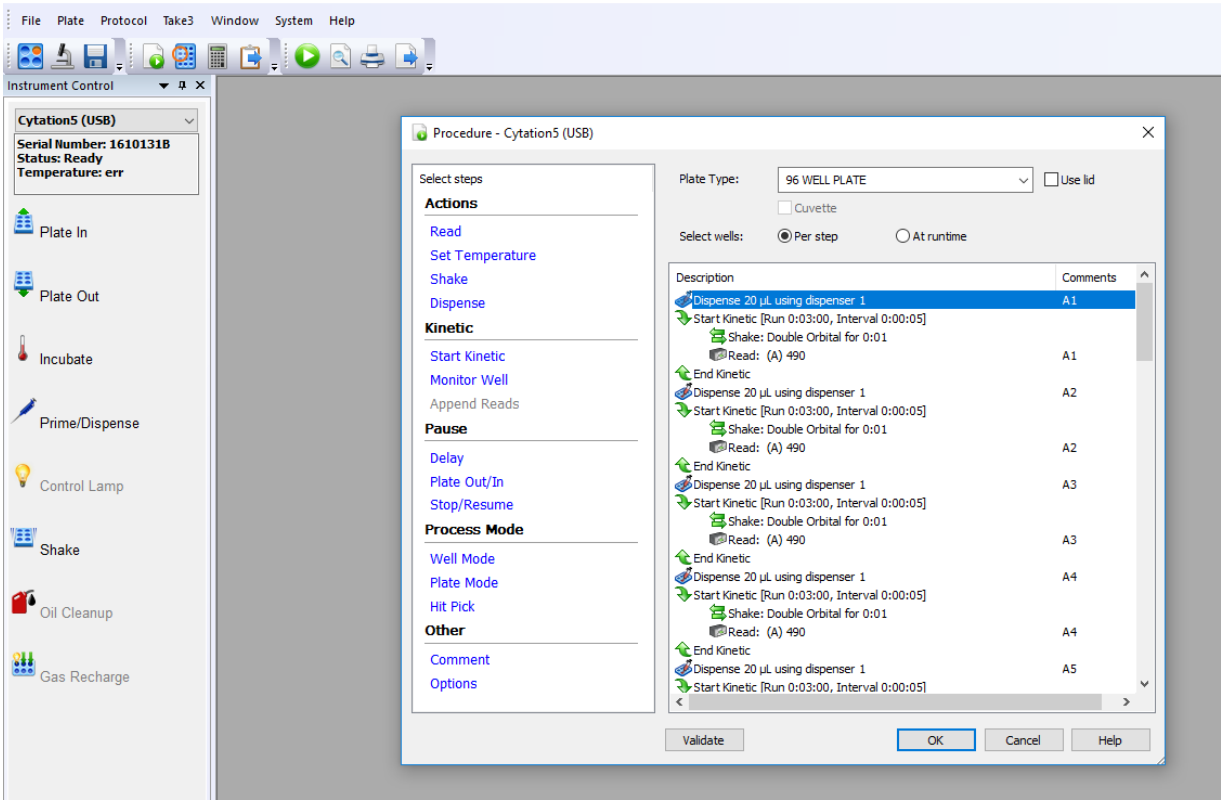


Figure 4 Biotek program for Cupredoxin assay

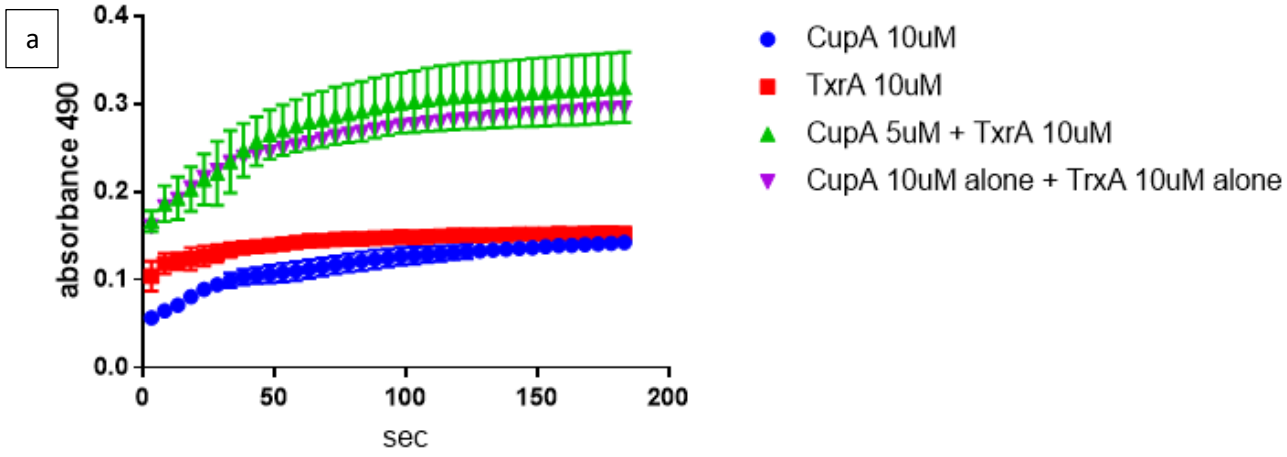
4 Results

4.1 Detecting Thioredoxin's Effect on CupA's Ability to Facilitate Copper Reduction

Although it was known from the microarray data that thioredoxin expression was upregulated under copper stress, the precise mechanism of thioredoxin effect on CupA to facilitate reduction

of copper had not been determined. Thus, we sought to elucidate if thioredoxin worked synergistically with CupA to reduce copper.

In this experiment, we tested thioredoxin's ability to reduce copper with and without various concentrations of CupA. The results were analyzed at 490 absorbance for every 5 seconds of time interval (Figure 5). We observed that thioredoxin alone was able to reduce the copper whereas the combined effect of both was not able to reduce to the copper. We also compared the results with different concentrations (Figure 5 a, b, c) which showed that the initial absorbance was too high at the lower concentration. Later, to get more accurate results, the blank was included. The readings obtained from the TrxA, CupA and combined had the blank subtracted (BCS and the copper injection). From the results, we were able to see the reduction with CupA alone at the initial point where as we were not able to see the additive reduction along with the thioredoxin. Taken together, we were not able to see the synergistic effect.



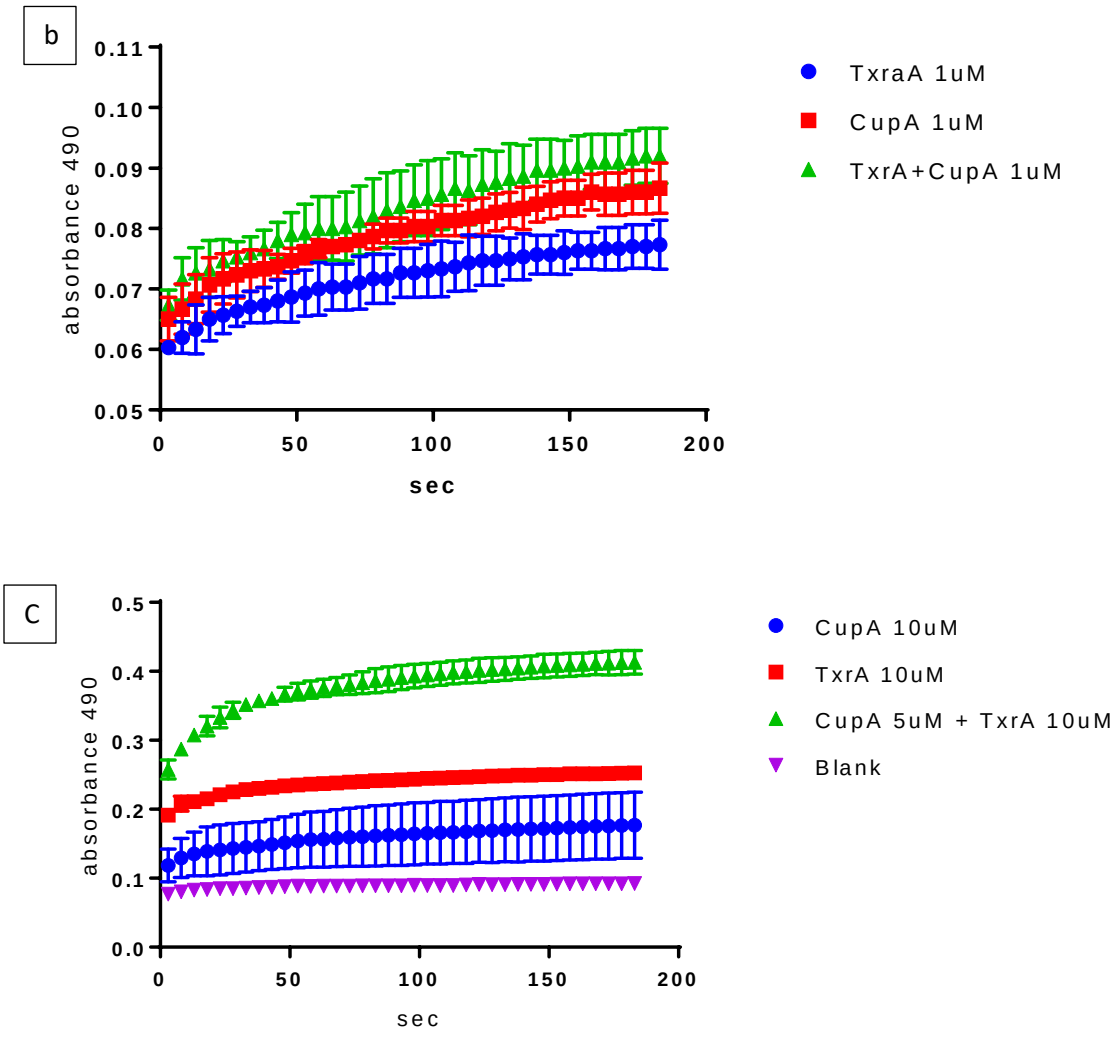


Figure 5 a) Effects of thioredoxin with CupA at 10uM b) Effects of thioredoxin with CupA at 1uM c) Effects of thioredoxin with CupA at 10uM along with the blank.

4.2 Thioredoxin Effects with the Help CupA Double Mutant (DM)

Next, we wanted to examine the thioredoxin role in copper reduction with the help of CupA DM. The CupA structure contains two Cu^{1+} atoms one coordinated by C74 and C111 (high affinity binding site) and the one coordinated by C74, M113 and M115 (low affinity binding site). We mutated the residues in the protein and then made a double cysteine mutant. This double mutant was unable to facilitate the reduction of Cu^{2+} to Cu^{1+}. The Cupredoxin assay revealed that

thioredoxin along with the CupA DM was not able to show the synergistic effect in the reduction of copper (Figure 6 a, b). We also tested the experiment for varying concentration of CupA DM and thioredoxin.

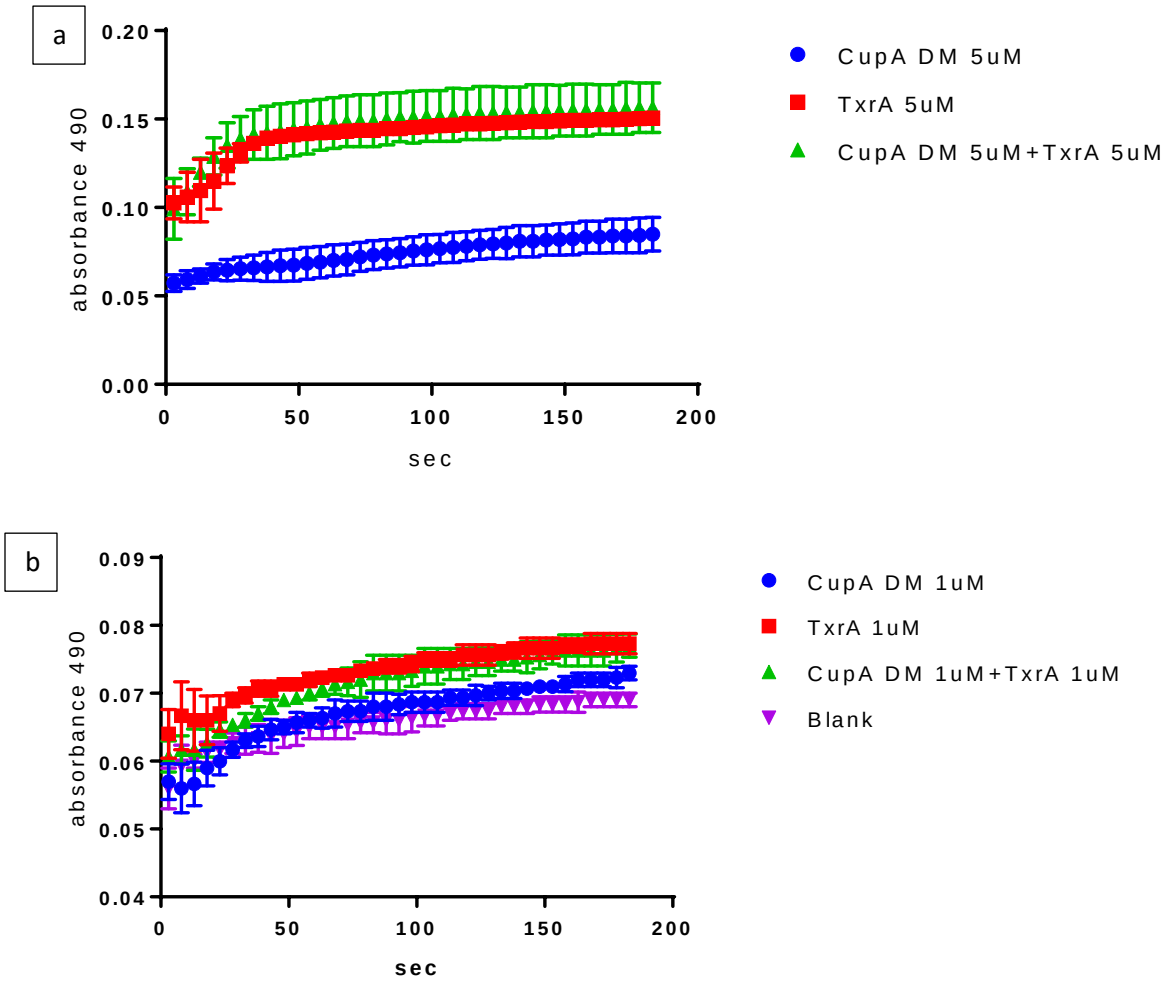


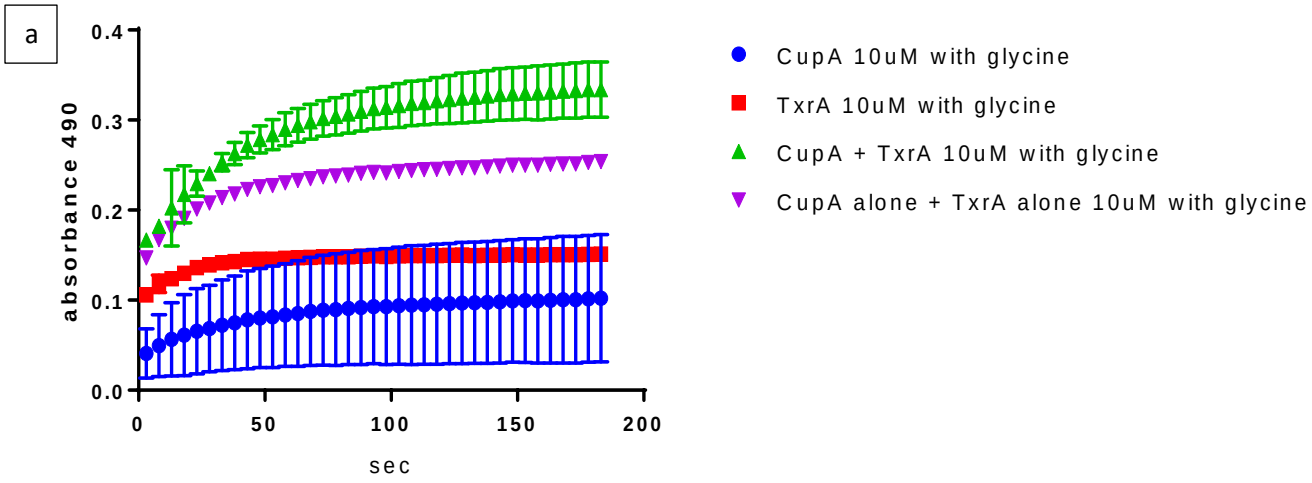
Figure 6 a) Effects of thioredoxin with CupA DM at 1uM b) Effects of thioredoxin with CupA DM at 5uM.

4.3 Use of Reducing Agent Glycine

In the previous assay methods, no external reducing agent was used. Previous studies found that glycine can be used as reducing agent for the copper (Saswata Bose, 2012). Furthermore, because

of the low price and non-hazardous features of glycine helps to contribute as a suitable reducing agent for the bulk production of graphene (Saswata Bose, 2012). It was also seen that the reduction could occur in mild condition which was confirmed by Raman Spectroscopy and transmission electron microscopy (Remko M1, January 5, 2006).

Thus, we sought to use the glycine as reducing agent as it would not bind the copper instead of DTT which would bind the copper. The assay was carried to study the reaction, the results suggested that even after adding the reducing agent we could partially explain the reduction of copper. However, with glycine we were still not able to see the synergistic effect in the curve (Figure 7). Within the comparable test we also compared the effects from the two different concentrations, which nonetheless had no synergistic effect (Figure 7).



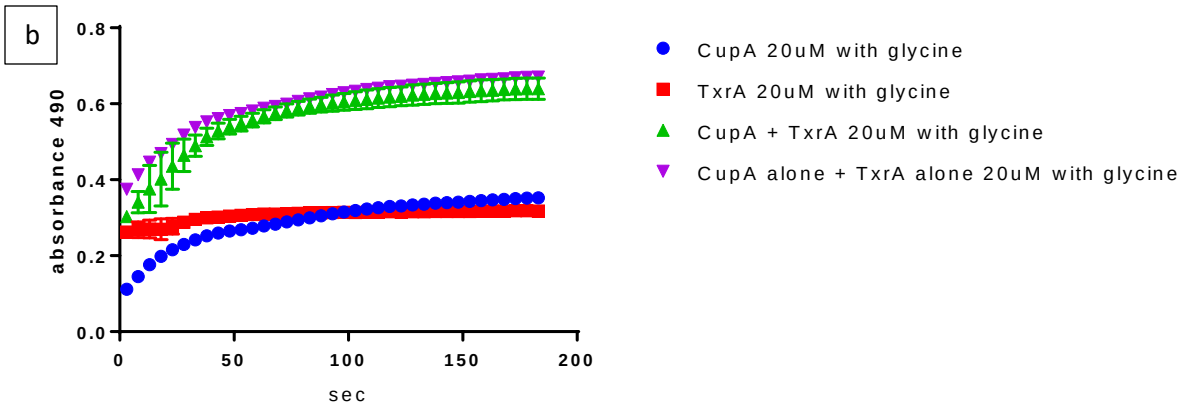
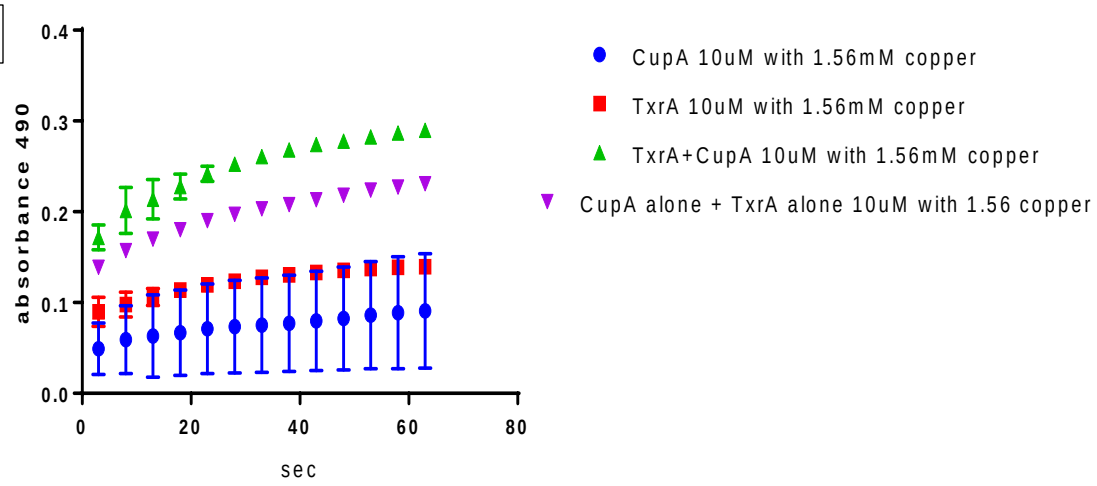


Figure 7 a) Effects of thioredoxin with CupA and Glycine at 10uM b) Effects of thioredoxin with CupA and Glycine at 20uM.

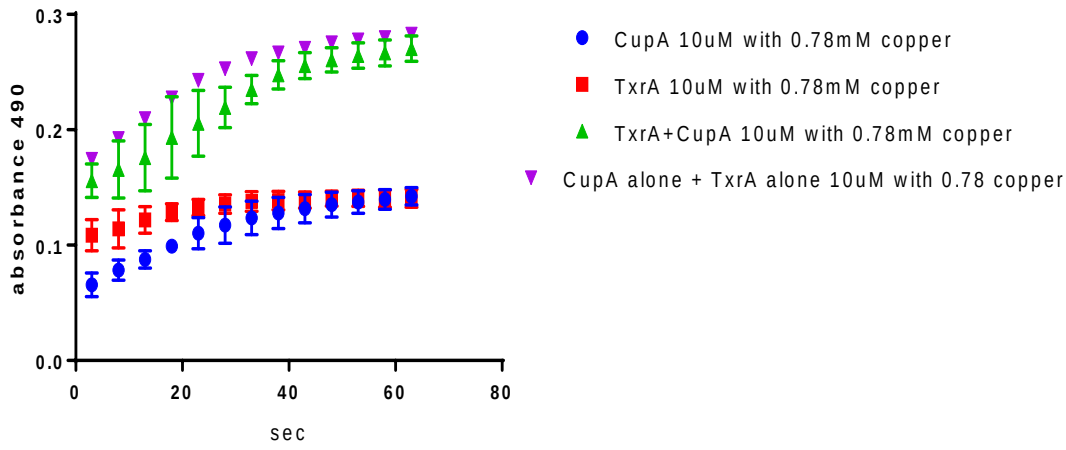
4.4 Varying Copper Concentrations

The interaction between the copper and protein are very stable. The binding of the BCS and the cuprous ion effectively removes the weakly chelated peptides in the reaction. Thus, to determine if copper is affecting in the reaction, we thought to work with different concentrations of copper. First, we assessed the effect of copper by using higher and lower copper concentrations. The results from these concentrations of copper did not lead to any change of previous conclusion that there was no synergistic effect between thioredoxin and CupA (Figure 8).

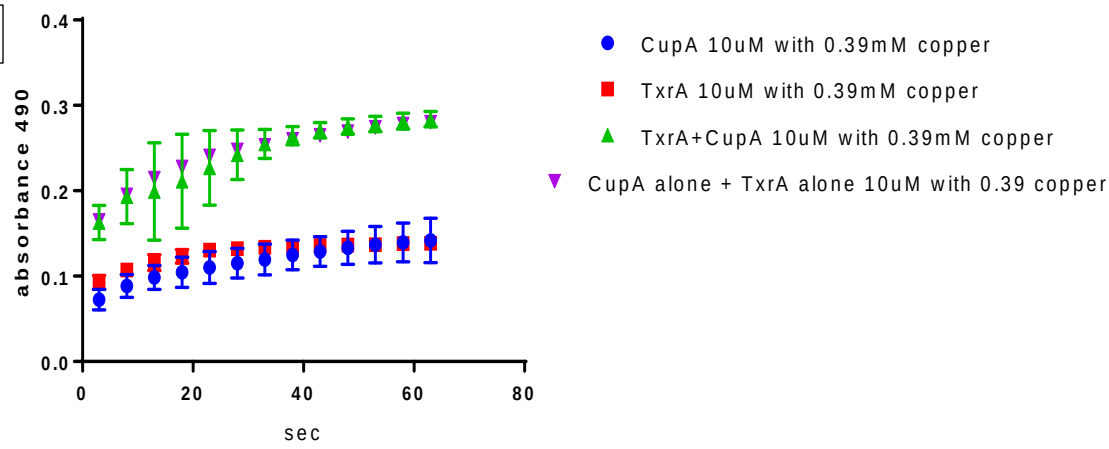
a


absorbance 490
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
sec
CupA 10uM with 1.56mM copper
TxrA 10uM with 1.56mM copper
TxrA+CupA 10uM with 1.56mM copper
CupA alone + TxrA alone 10uM with 1.56 copper

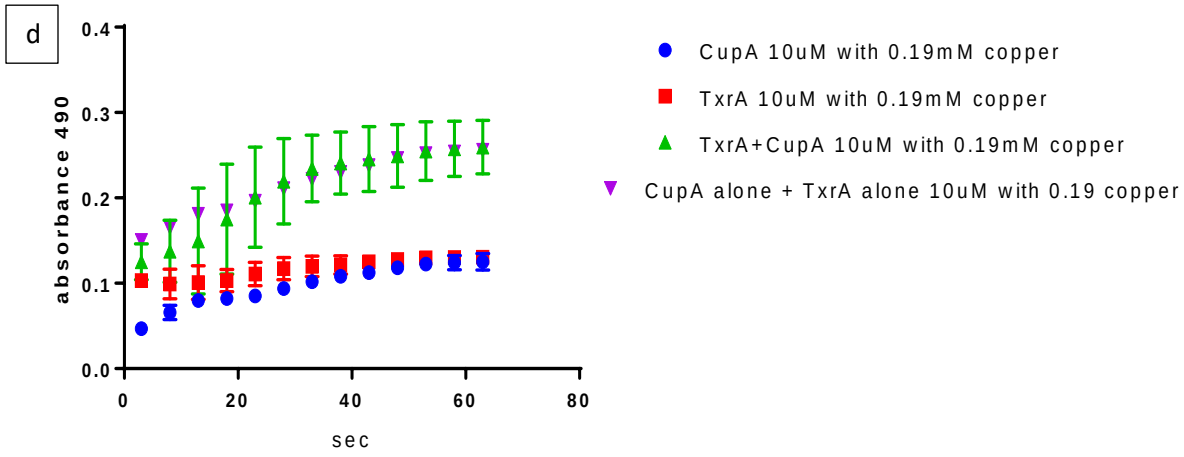
b


absorbance 490
0.3
0.2
0.1
0.0
0
20
40
60
80
sec
CupA 10uM with 0.78mM copper
TxrA 10uM with 0.78mM copper
TxrA+CupA 10uM with 0.78mM copper
CupA alone + TxrA alone 10uM with 0.78 copper

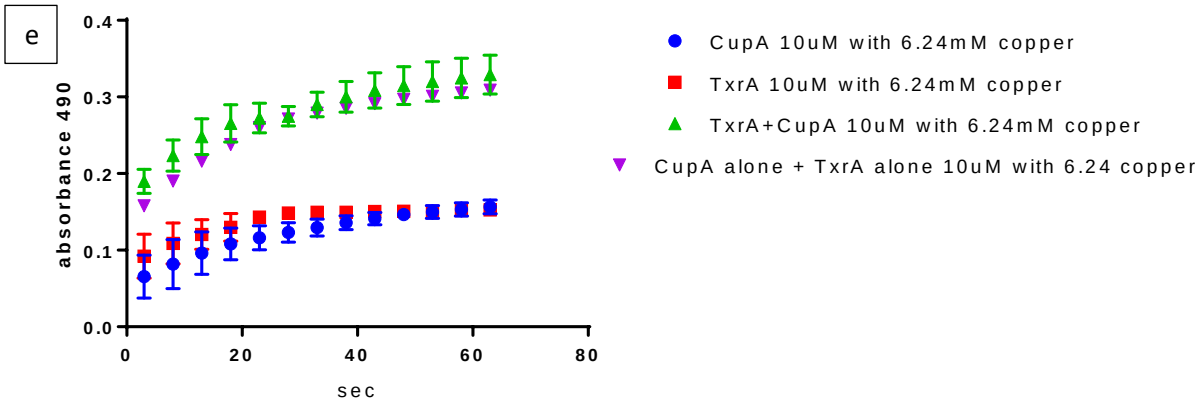
c


absorbance 490
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
sec
CupA 10uM with 0.39mM copper
TxrA 10uM with 0.39mM copper
TxrA+CupA 10uM with 0.39mM copper
CupA alone + TxrA alone 10uM with 0.39 copper

d


absorbance 490
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
sec
CupA 10uM with 0.19mM copper
TxrA 10uM with 0.19mM copper
TxrA+CupA 10uM with 0.19mM copper
CupA alone + TxrA alone 10uM with 0.19 copper

e


absorbance 490
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
sec
CupA 10uM with 6.24mM copper
TxrA 10uM with 6.24mM copper
TxrA+CupA 10uM with 6.24mM copper
CupA alone + TxrA alone 10uM with 6.24 copper

f

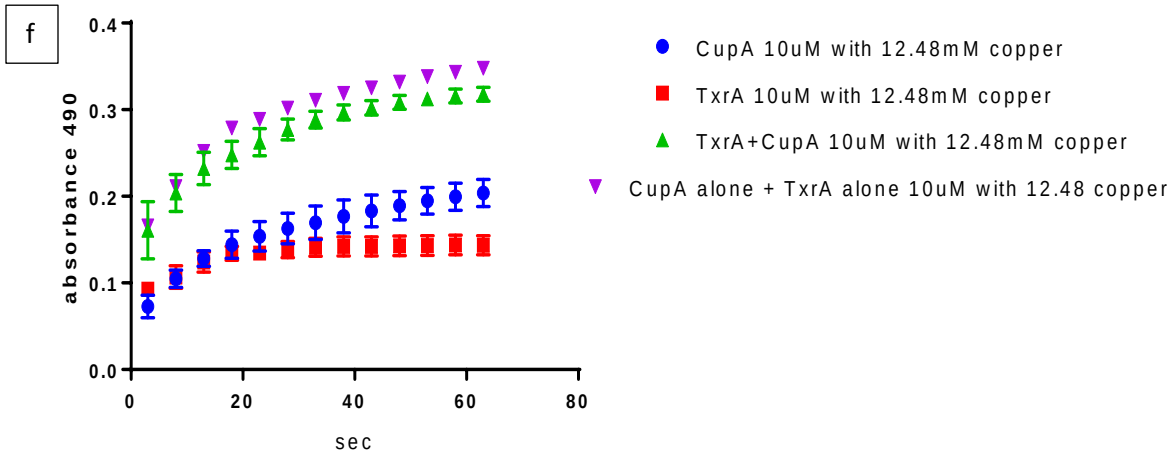
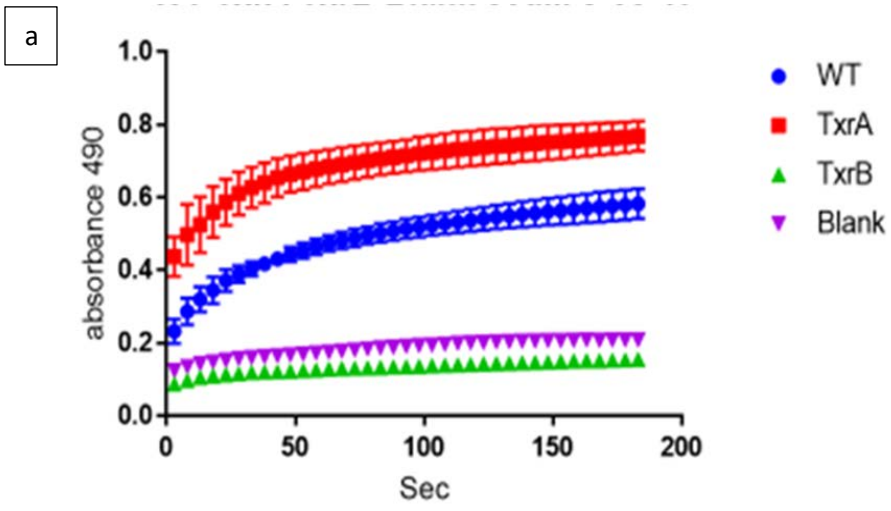

absorbance 490
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
sec
CupA 10uM with 12.48mM copper
TxrA 10uM with 12.48mM copper
TxrA+CupA 10uM with 12.48mM copper
CupA alone + TxrA alone 10uM with 12.48 copper

Figure 8 Copper injection at 4-fold lower and 2-fold higher concentration a) at 1.56mM b) 0.78mM c) 0.39mM d) 0.19mM e) 6.24mM f) 12.48mM.

4.5 Effects of TrxA and TrxB and CupA DM

Thioredoxin reductases (TrxB) are oxidoreductases which are required for the reduction of the active site disulfide in thioredoxin. In several bacteria thus TrxB is responsible for maintaining the pool of reduced and active thioredoxin (Arner ES, 2009). With this function of TrxB we next sought to study the combined effects of TrxA and TrxB with the CupA, from the results we were able to see that TrxB was not contributing in the reducing of copper with TrxA and CupA (Figure 9a). Later, we wanted to see if TrxB along with CupA DM would have any effect in copper reduction, the results showed that there was no any change from all the previous experiments that there was no synergistic effect even with the TrxB, TrxA and CupA DM (Figure 9b).



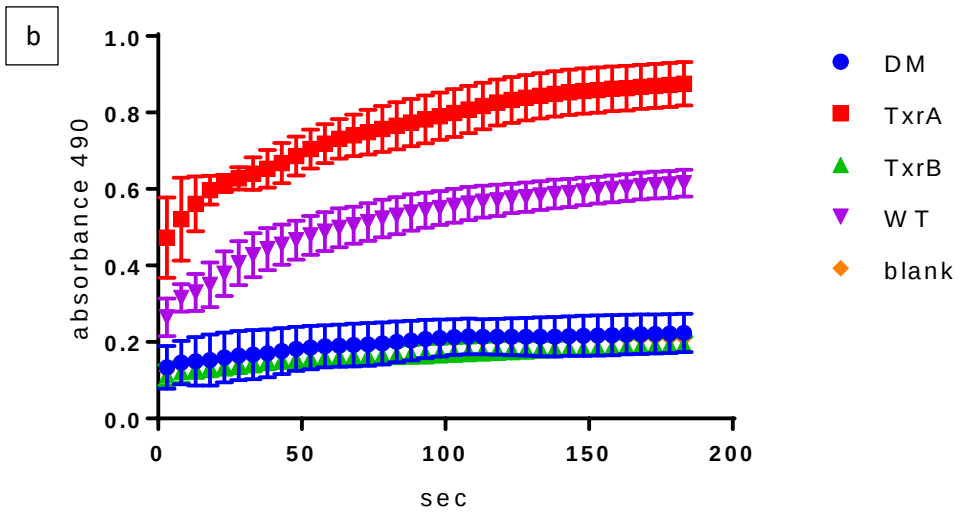


Figure 9 a) Effects of thioredoxin reductase, thioredoxin with CupA (WT) b) Effects of thioredoxin reductase, thioredoxin with CupA DM.

5 Discussion

In this study, we examined if thioredoxin worked synergistically with and CupA to reduce copper. Based on the various trails of experiments, we conclude that there were no synergistic effects between the two proteins.

From the previous studies thioredoxin acts as a reductant in many cellular reaction, such as ribonucleotide reactions, transfer the reducing equivalents from cytoplasm to cell via membrane bound oxidoreductases and it helps to keep the cysteine residues in its secretory precursor and cytoplasmic proteins in cytosol reduced form (Holmgren E. S., 25 December 2001) (Holmgren A. , July 1985). Inside the cell proteins contain the free sulfhydryl groups and rare disulfides. The major universal disulfide reductase is responsible for maintaining proteins in their reduced state is thioredoxin which is reduced by electrons from NADPH via thioredoxin reductase (Figure 10) (Jones DP, 2008 Oct) .

Initially, from our microarray data, we observed that the thioredoxin genes were turned up. Thioredoxin system is one of the important antioxidant systems in mammalian cells, sustaining a reducing environment by catalyzing the electron flux from nicotinamide adenine dinucleotide phosphate through thioredoxin reductase to thioredoxin, which helps in reducing the target protein with the use of highly conserved thiol groups (Laurent TC, 1964 Oct) (Samuel Lee, 2013 Apr 1).

Thioredoxin was used in our study to study copper reduction. In *S. pneumoniae* thioredoxin was helpful in reduction of hydrogen peroxide (H_2O_2) and catalyzing the reaction and thereby preventing the oxidative stress from copper (Samuel Lee, 2013 Apr 1).

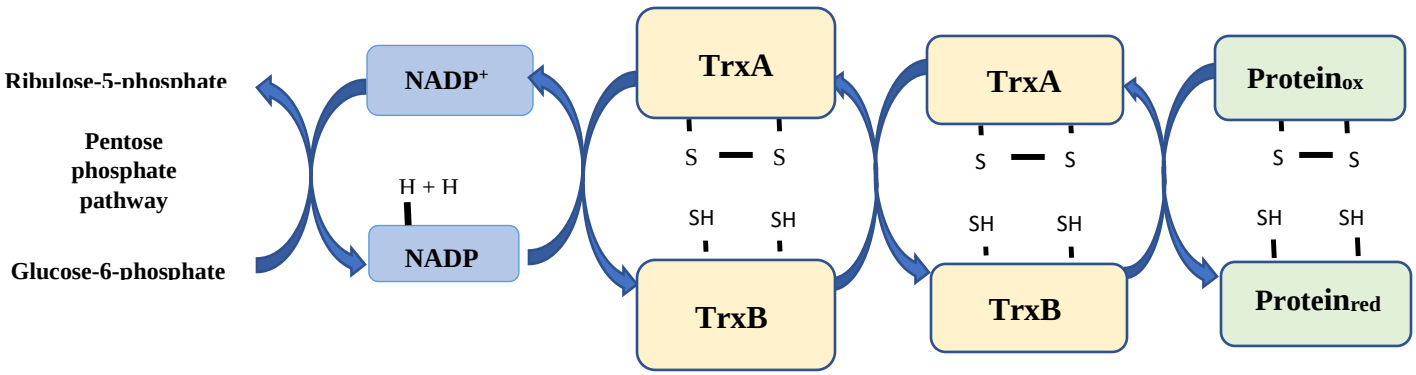


Figure 10 Redox cascade of the Trx system. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)+H+ is generated by the pentose phosphate pathway. NADPH+H+ reduces oxidized Trx reductase (TrxA), which regenerates the pool of reduced TrxB. Reduced TrxB contributes to maintaining a reducing environment for several different proteins (Samuel Lee, 2013 Apr 1).

Secondly, in our Cupredoxin assay the copper chaperon CupA has an ability to reduce the copper and release Cu^{1+} for BCS chelation. In our proposed copper reduction model of CupA, Cu^{2+} is chelated by CupA and reduced to Cu^{1+} (Figure 3). This process does not negate CupA's ability to bind Cu^{1+}. From the previous studies the reduction process is facilitated by C111 and C74, as shown by the inability to C74A C111A protein variant to either facilitate reduction of Cu^{2+} or restore CopY binding to DNA in the presence of Cu^{2+} (Miranda J. Neubert, 2017 Sep-Oct). CupA's crystal structure denotes C74 and C111 as high-affinity site 1 for the binding of Cu^{1+} and C74, M113 and M115 as low-affinity site 2 for the binding of Cu^{1+} (Yue Fu H.-C. T.-T., 27 January 2013). We have observed that the copper reducing event occurs in conjunction with site 1 and the resultant Cu^{1+} atom is passed to site 2 to facilitate export via CopA (Figure 3).

Finally, in our Cupredoxin assay the BCS has been used as for copper chelator. From different studies it's known that the chelating agent BCS will form stable complexes with the monovalent copper (Cu) which has typically absorption at 450-490nm (D. Huang, 2005). BCS protects the cysteine from oxidation to cysteine in a perfusion buffer (CarlosCampos, 2009). Our results showed that there was no synergistic effect, which may be thought that BCS did not inhibit the autoxidation of cysteine. However, it is seen from the results even after varying the concentration of copper the BCS was still not able to show any synergistic effect.

Hence, overall results for the copper reduction with thioredoxin and copper chaperon CupA was failed to show the synergistic effect in copper reduction. These findings and our data can give an insight in the field copper toxicity in *S. pneumoniae.*

6 Future Directions

Taken together, the observed results from the current experimental design we were able to see that thioredoxin and thioredoxin reductase along with copper chaperone CupA had no synergistic effect in reduction of copper. These findings helped to have insight for the future direction, where we can move forward by, trying if inhibitors could have an additional effect in copper reduction. Understanding the individual effects of TrxA, TrxB and CupA from the previous experiments would make a powerful approach in studying the combined effects of all three proteins.

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“Optimization of antibiotic assay using Smarticle based method”

By

Preetham Hosur

A report submitted to the faculty of the

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In partial fulfillment of the requirements

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Contents

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1 BACKGROUND

The overuse of antibiotics has driven the evolution of the drug-resistant strains, an increasing problem worldwide (World Health Organization, 2017). Resistance to antimicrobial agents has resulted in the mortality from treatment failures and increased health care cost (Sengupta S, 2013). Infections with multidrug-resistant bacterial organisms (MDRO), i.e. bacteria that are resistant to one or more classes antimicrobial agents, are spread in hospitals with increasing frequency (Spellberg B, 2008). Every year in United States, at least 2 million people acquire a serious infection with multidrug resistant bacteria and at least 23,000 people die each year because of this antibiotic resistant infection (Center for Disease Control and Prevention, 2013).

The World Health Organization (WHO) are predominantly concerned about the loss of effective antibiotic treatment that will not only lose the ability to fight against the infection but also weakens patients and increases the complication to cure the infection (Aboelela S, 2006). Also, some major treatments are dependent on the antibiotics to fight the infection after the surgery to keep the patients safe and combat. Inability to treat patients with efficient antibiotics could negatively affect patients access to effective treatment and decrease advancement in medical treatment. (Centers for Disease Control and Prevention, 2012).

Antimicrobial drug development is slowing with the increase in antimicrobial resistance (Larson, 2015). To conserve the antibiotics we have, hospitals have initiated antimicrobial stewardship programs, where clinicians and doctors work together find the optimal selection, dosage and duration of antimicrobial treatment which results in the best clinical outcome for the treatment or prevention of infection (Gerding DN, 2001). The goal of this program is to optimize the antibiotic use is that they help in cost saving by choosing the fewer dose and less expensive antibiotics.

Antimicrobial stewardship helps guide the appropriate use antibiotics in conjunction with Antimicrobial Susceptibility Tests (AST), which in turn helps in reduction of antimicrobial resistance (Philippe Morency-Potvin, 2017)

Current AST methods need three or more days from the time of sample collection to result reporting, resulting in patient health deterioration and antibiotics mistreatment (Clinical and Laboratory Standards Institute, 2017). The objective of susceptibility testing is to predict the specific antimicrobial agent and the minimum inhibitory concentration (MIC) of that specific antimicrobial agent to treat a patient's infection. Susceptibility testing is useful for bacterial species that are not typically vulnerable to drugs of choice because of potential acquired resistance mechanisms (e.g. members from the *Enterobacteriaceae, Pseudomonas species, tests, and Staphylococcus species, Enterococcus species, Streptococcus pneumoniae, Herophilus influenzae, and Neisseria gonorrhoeae*) (Ferraro J. H., 1998).

2 CURRENT ANTIMICROBIAL SUSCEPTIBILITY TESTING METHODS

The two critical functions of susceptibility testing are the detection of resistance and the quantitative measurement of susceptibility to antimicrobial agents that may have a direct therapeutic relevance (Murray P.R, 1983).

Various methods of antibiotic susceptibility testing are:

1. Quantitative Methods
2. Qualitative Methods

2.1 Quantitative Methods

In this method bacterial isolates are exposed to some range dilutions of several antibiotics. The lowest dilution of antibiotic that has inhibited the growth of bacteria is considered as the MIC. This can be performed on both broth as well as the agar.

a) Broth dilution methods
    - Macrobroth dilution MIC test:
    - Microbroth dilution MIC test
b) Agar dilution methods

2.1.1 Broth dilution methods

2.1.1.1 MACROBROTH DILUTION MIC TEST

In this method the two-fold serial dilution of antibiotics diluted in 1-2 mls of growth media are made in test tubes from zero to maximum clinically relevant concentration. The inoculum density of the organism to be tested is standardized with 0.5 McFarland turbidity standard or approximately the final inoculum of 5×10^5 CFU/ml. The lowest concentration of antibiotics that inhibits the complete growth of organism is considered as the MIC (Ferraro L. B., 2009).

2.1.1.2 MICROBROTH DILUTION MIC TEST

In this method a 96 well polystyrene plate is used and filled with small volumes of two-fold dilution of various antibiotics and carried out the same method as in the macrobroth. Following the overnight incubation at 35°C plates are examined for the bacterial growth visible bacterial growth as a seen by turbidity. The lowest concentration of the antibody that prevented the growth represents the MIC (Ferraro L. B., 2009). Using automated instrumentation can help in standardizing the reading end points and produce susceptibility test results in a shorter period than

manual reading because these instruments are embedded with special feature of sensitive optical detection which helps in the detecting the irregularity in the bacterial growth (Richter SS, 2007).

2.1.2 Agar dilution methods

A serial two-fold dilution of the antibiotic is prepared in Mueller-Hinton agar. A loopful of organisms is inoculated on the agar surface and is incubated at 37°C overnight. The lowest concentration of antibiotic that inhibits visible growth on surface of agar is taken as the MIC (Petra Luber, 2003).

2.2 QUALITATIVE METHODS

2.2.1 Disk Diffusion Test

The BD Sensi Disc (Becton Dickinson GmbH) is a commercial product in which a standardized concentration organism is spread on the agar plate and then the paper disk of an antibiotic concentration or the metallic cylinders are placed on the agar medium. If the organism is susceptible to the antibiotic, it does not grow around the paper disc or the cylinder forming the zone of inhibition which can be measured by visual inspection. From the plate it is seen that if the isolates colonies are grown, then the zones are measured to nearest millimeter. If the inoculum is too light, then the test has to be repeated (BD Diagnostic Systems, 2005).

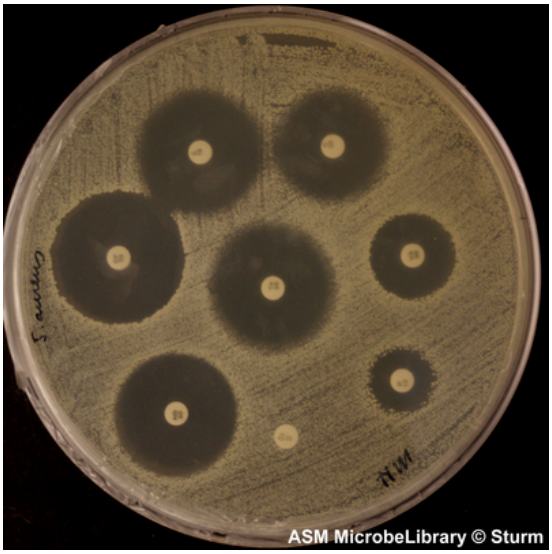

Figure 1 Disk diffusion Plate (Hudzicki, J, 2009)

2.2.2 E-Test Strips Method

The Etest (bioMérieux AB BIODISK) is a commercial product for use in the United States. The test consists of a predefined gradient of antibiotic concentration on a plastic strip and use to determine the MIC of antibiotics, antifungal agents and antimycobacterial agents. These strips are placed in the radial fashion on the agar plate which has been inoculated with a standardized organism suspension. After overnight incubation, the tests are read by viewing the strips from top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip (bioMérieux, Inc, 2006).

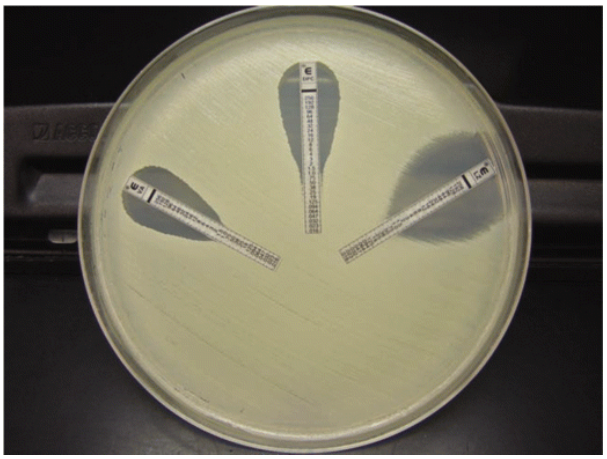

Figure 2 Etest gradient diffusion method (Ferraro L. B., 2009)

2.2.3 Vitek 2 System

The Vitek 2 system (bioMérieux) is an improvised and computerized testing platform which helps to reduce the human effort in set up time. The major component of the system is it uses the plastic card which are predefined with antibiotics in them. It is very similar to the test conducted using 64 well format. This system helps in continuous monitoring of the bacterial growth during entire incubation period. These cards allow the testing of both gram positive and gram-negative bacteria (bioMérieux, Inc., 2017).

2.2.4 Sensititre ARIS 2x

The Sensititre ARIS 2x (Thermo-Fisher Scientific) is an automated overnight incubation and reading system with 64 panel capacity. The test panels are standard 96 well microdilution plates which can be inoculated with Sensititre Auto inoculator. The instrument is made up with heated carousel which ensures optimum growth condition by incubating all plates individually and it also helps to exclude the repeat tests. The results are interpreted after the incubation for 18 to 24hrs by fluorescence measurement (TREK Diagnostic Systems, 2013).

3 SMARTICLE TECHNOLOGY

Smarticle technology is a novel and innovative method that can be used to perform the AST method which helps in identifying the multi resistant organisms (MDROs). The advantage of this method is that it evaluates antibiotics susceptibility directly from clinical sample without the need for culture preparation or sample preparation. The Smarticle can be designed to any specific species, genus and family of bacteria.

This technology consists of a modified plasmid with bacterial luciferase *luxAB* reporter system which is packaged with the pathogen-specific bio particles. This results in a Smarticle. Smarticles infect live target bacteria, which then to produce luminescence (Figure 5).

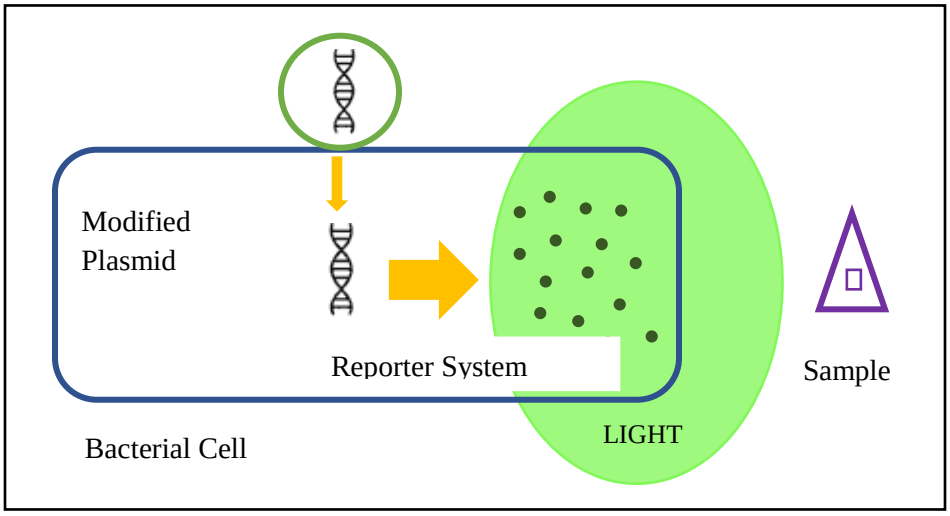

Figure 3 Smarticle Technology

While testing for susceptibility, antibiotics are added to the assay. Bacteria are targeted by the Smarticle and if the target bacteria are susceptible to the antibiotic, then more bacteria in that sample are dead and less are transduced, which results in less light. Resistant bacteria, on the other

hand, are less affected by the antibiotic and therefore can produce the light and continue growing. This helps in detecting the drug resistant organisms and determine what therapies will work. In just a few hours the test can reveal which drugs works the best. This luminescence output, measured in Relative Light units (RLU), is used to determine appropriate antibiotic treatment. RLUs are compared between wells to determine susceptibility or resistance to specific antibiotics (United States Patent No. US 9,388,453 B2 , 2016).

In my study we are trying to develop a rapid identification/AST test that reduces this time to less than the current methods days using Smarticle technology. This Smarticle technology approach is different as it is fast to deliver time critical information in few hours directly from the patient's sample. The technology helps guide health care providers to use the right drug on the first dose. According to FDA guidance documents on developing new ASTs, this new Roche assay method and its optimization will be evaluated by analyzing categorical agreement (CA) which is defined by the percent of isolates producing the same categorical result the existing reference method. These categorical results are either Susceptible(S), Intermediate (I) or Resistant(R). The assay will also be compared to the gold standard to evaluate essential agreement (EA) which is defined when the MIC is within the ±1 two-fold dilution under the evaluation with the reference method.

4 METHODS AND MATERIALS

Different isolates with different levels of RLU output and SIR profiles were selected to test against the reference method. A single 96-well microdilution plate was used with different concentrations of antibiotics including one reference well in it with no antibiotics. The reference well is considered as a positive which is used as a threshold during the assessment of the result. Then the calculated amount of antibiotics and the isolates were added into the 96 well plates using automated liquid

handling instrument (Hamilton STAR). The Smarticle was then added to measure bacterial growth and viability. Finally, a SpectraMax-L Analyzer was used to read the plates and to determine maximum RLU for different strains of isolates.

These above steps are repeated for each different condition of assay workflow. This workflow was tested in duplicate. Performing assay in duplicates is suggested in the workflow to check for pipetting error, multiple skipped wells, contamination or other potential process error problems. The details of the conditions and the method cannot be discussed in more detail because the assay is the intellectual property of Roche Molecular Solutions.

Conditions were compared by analyzing the difference in the percent categorical and essential agreement. Categorical agreement is defined by the percent of isolates producing the same category result (S I R) as compared to the standard/reference/existing method. Essential agreement is defined by the percent of samples that have a MIC with or within ±1 two-fold dilution of the MIC established by the reference method. The performance characteristics of categorical agreement is the percent of isolates producing the same category result (S I R) as compared to the standard method. The interpretive criteria for the MIC is based on the breakpoints determined by CLSI guidelines. These breakpoints help in providing the basis for categorizing the results of *in vitro* susceptibility tests into predictable outcomes.

The breakpoints of AST results are reported by the antibiotic sensitivity interpretation: S (Susceptible), I (Intermediate) or R (Resistant) (Clinical and Laboratory Standards Institute, 2017). A susceptible (S) result implies that the organism is inhibited by the concentration of the drug that is attained using the recommended dosage. An intermediate (I) result implies that the organisms are inhibited only by the maximum recommended dosage. A resistant (R) result implies that the

organisms are not inhibited by any clinically relevant drug levels. When the current test method produces a different result from the established reference method, those discrepancies are divided into three types: minor error, major error, and very major error. Discrepancies are categorized as minor errors when the reference result is R or S and assay result is I; reference result is I and assay result are R or S. Discrepancies are major errors when the reference result is S and assay result is R; very major errors are results where the reference result is R and assay result is S.

5 RESULTS

This data obtained from SpectraMax was analyzed by using statistical software called JMP. The MIC is determined based in the RLU. The details cannot be discussed in more detail due to legal restrictions from Roche Molecular Systems.

The results also showed a comparable percentage of categorical agreement, error rate, and essential agreement between the test conditions. In our study the results from the antibiotics sample wells were compared to the control plates. The overall results show that the test conditions tend to give lower standard deviations, less variability and lower number of errors for the all the tested isolates when compared to control data. Due to Roche legal restrictions, the details of the results cannot be thoroughly discussed.

6 CONCLUSIONS

The overall study shows test method a considerable promise in adopting this new optimization parameter for assay development study. This method also has added advantage that include the economy of the reagents and the space because of scaling down the test, also the reproducibility of the result due to the preparation of large of number of wells from the same antibiotic dilution

series. The two conditions also have similar minor, major and very major error rates. These errors play a major role because there are features relevant to antimicrobial treatment. The very major error is worse than any other errors because the assay result is susceptible, but the organism is resistant then the patient might be inappropriately treated, or a physician may choose a wrong medicine which leads to death of the patients. Whereas in the major error the patient will not be treated inappropriately but the categorical agreement would be wrong, and they can survive. The patients would be prescribed with over dosage of drugs. At the later stages of the treatment the organisms become resistant to this over dosage which is again not curable among that community causing a serious threat against the infection. The minor error is not serious as the errors fall under intermediate category where organism is inhibited at maximum dosage where patients can survive.

This method helps in the luciferase expression producing light, which is used to quantify the number of bacterial cells and allow the rapid AST. Furthermore, this method assesses the antibiotic susceptibility directly from the clinical samples without the need for the sample preparation or the organism isolation. This will give a quick rule-in appropriate antibiotic therapy within hours, so that the healthcare providers will know the best treatment to use at the earliest possible moment.

Using Smarticle technology, were we able to analyze gram-negative bacteria for susceptibility to multiple classes of antibiotics and the results are obtained within 4hrs without the need for complex sample preparation.

From the overall results we were able to see that with the introduction of the new condition, the errors were minimized which will help the doctors to prescribe the right antibiotic treatment at disease onset without causing a threat to life. The results were obtained within few hours which

potentially helps in slowing the evolution of antibiotic resistance and increasing the efficacy of antibiotic stewardship programs.

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