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Investigating Antibiotic Resistance Levels of *Salmonella* Internalized in Lettuce Leaves

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Abstract

Contamination of food crops by the human pathogen *Salmonella* is a food safety threat worldwide. Though using treated wastewater for irrigation is a sustainable practice, it may introduce trace levels of *Salmonella* that may contaminate food crops. *Salmonella* could develop resistance to antibiotics present in wastewater. The overall goal of the project is to increase the understanding of the public health risk associated with the use of treated wastewater to irrigate food crops. The objective of this particular study is to determine the antibiotic resistance level of *Salmonella* internalized in lettuce leaves.

In this experiment, thirty-six plants of the lettuce (*Lactuca sativa*) cultivar Green Salad Bowl were grown hydroponically in Hoagland growth medium and harvested at 21, 35, and 48 days. Five days before each harvest, solutions containing the antibiotic oxytetracycline (OTC) and the bacteria *Salmonella* enterica serovar Infantis were added to the hydroponic growth medium. At each harvest time, bacteria extracted from the lettuce leaves were quantified on XLD agar plates amended with 1, 2, 4, 8, 16, or 32 mg/L OTC. After an 18-20 hour incubation period, colonies that turned black were enumerated. By comparing the number of colonies grown on different plates, the antimicrobial resistance level of the internalized *Salmonella* was determined. Results show that the

antibiotic resistance levels of *Salmonella* grown in LB media not receiving OTC, *Salmonella* internalized lettuce not receiving OTC, and *Salmonella* internalized in lettuce receiving OTC were all at 32 mg/L OTC. In addition, no significant differences were observed among different lettuce growth stages.

1. **Introduction**

Both climate change and the growing world population have impacted earth's freshwater resources (Zakar, 2012). As these factors cause freshwater to become increasingly scarce worldwide, many regions resort to using treated wastewater directly for domestic, industrial, and agricultural uses (Hamilton, 2006). However, the detection of trace level antimicrobials and pathogens have been regularly reported in wastewater effluents and in recycled wastewater (Fatta-Kassinos, 2011). Due to prolonged exposure to antimicrobial residuals in wastewater, some bacteria, including human pathogens such as *Salmonella*, develop resistance to these antimicrobial compounds. In the presence of low levels of antimicrobials, bacteria have shown the ability to acquire antibiotic resistance genes (Gal-Mor, 2010).

When treated wastewater is used as a source of irrigation water, antibiotic resistant (ABR) pathogens such as ABR *Salmonella* may be transmitted to food crops. *Salmonella* has been shown to be internalized into lettuce leaves following wastewater irrigation (Levantesi, 2012). The trace levels of antibiotics from wastewater irrigation have also been found in lettuce (Boxall, 2006). Furthermore, the environmental conditions inside plant leaves may affect the antibiotic resistance levels of the internalized *Salmonella*.

This experiment was designed to explore the antibiotic resistance levels of a model human pathogen *Salmonella* internalized in lettuce plants. Antibiotic resistance levels are defined as the concentration of antibiotic at which the ABR bacteria can no longer survive. Determining resistance levels for particular bacteria is important in predicting the risk of proliferation of the bacteria in the environment. This experiment will lead to a better understanding of the fate and transport of *Salmonella* in the agricultural environment. Additionally, this work is beneficial because it provides information about the possible public health risks associated with the use of treated wastewater in food crop irrigation.

2. Literature Review

Water scarcity is an issue that is becoming increasingly more serious. Fresh water is becoming scarcer due to two main factors: population growth and climate change (Zakar, 2012). Global climate change could contribute to less precipitation, more drought, as well as degrade the quality of fresh waters (IPCC, 2001). With these two factors at play, it is necessary to find alternative means to provide water for various uses. Many regions worldwide are using recycled wastewater directly for domestic, industrial and agricultural uses (Hamilton, 2006). As agricultural irrigation accounts for about 75% of the world's freshwater withdrawal, using recycled wastewater for irrigation may potentially decrease the stress on freshwater resources (Qadir, 2006 and Zakar, 2012). Though irrigating with treated wastewater is a sustainable practice, there are some implications which must be considered when using it.

Treated wastewater effluent has been reported to contain trace quantities of contaminants such as pharmaceuticals and bacteria (Ahmed, 2013). Many types of bacterial pathogens, e.g. *Salmonella,* are also present in recycled domestic and agricultural wastewater. The bacteria are naturally found in feces of human and warm-blooded animals and can enter the water supply from either sewage or runoff from agricultural lands (Brenner, 1988). Specifically, past *Salmonella* outbreaks have been linked to both human and animal feces in wastewater (Berge, 2006). Many pharmaceuticals, e.g. antibiotics, are inefficiently metabolized by people or the animals that use them and can later be found in wastewater. Most of these chemicals are at such low concentrations in food crops that they present little health risk (Guardabassi, 2002). However, over time human pathogens in wastewater and in soil may develop antibiotic resistance in the presence of antibiotics.

Bacteria adapt and evolve to their surroundings when selective pressures such as temperature, pH, and predators are present (Sharma, 2014). Through natural selection many bacteria perish under these pressures, but others survive and proceed to have offspring that also have genes providing resistance to these pressures. This passing of genes from generation to generation is known as vertical gene transfer. There is also horizontal gene transfer, which is when bacteria acquire genetic materials from other bacteria of the same or different species. Genes coding traits that help the bacteria survive environmental pressures can be transferred by plasmids, transposons, or integrons (Devirgiliis, 2011). Antibiotics used to treat bacterial infections can be considered a selective pressure.

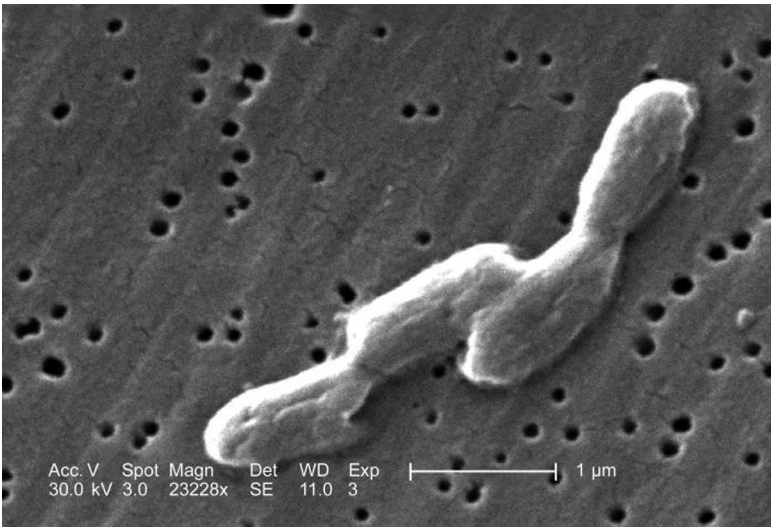

Figure 1. Image of *Salmonella enterica* serovar *Infantis* (Carr, 2005)

Antibiotic resistance is the ability for a microbe to not be effected by antibiotics, which were once used to kill them or control their growth. Antibiotic resistance levels are often described as minimum inhibitory concentrations (MICs) which is the lowest concentration of antibiotic needed to inhibit the growth of an organism (Mann, 1998).

During irrigation there is a risk that both of these antibiotics and pathogens in the wastewater may be taken up by food crops. The uptake of antibiotics by plants may influence plant development by disrupting the plant-microbe symbiosis. Plants may be used as vectors for some pathogens to live in until they reach a human host (Levantesi, 2012). Studies have shown *Escherichia coli* and *Staphylococcus aureus/methicillin-resistant S. aureus* (MRSA) can live in plant tissues (McMahon, 2006). Many *Salmonella* outbreaks today are caused by consuming raw produce such as leafy vegetables and uncooked fruits and vegetables (Center of Disease Control, 2009). Studies have been done to characterize bacterial adaptation to both natural and synthetic antimicrobials in crops, but still this phenomena is largely unknown and must be more thoroughly investigated (Dubois-Brissonnet, 2011 and Boxall, 2006). Plants are not optimal environments for *Salmonella*, which stresses the bacteria and may increase the likelihood of developing resistances to harsh environments, such as those with antibiotics present. The antibiotics accumulated inside the plant may also be considered a stress to the internalized *Salmonella*.

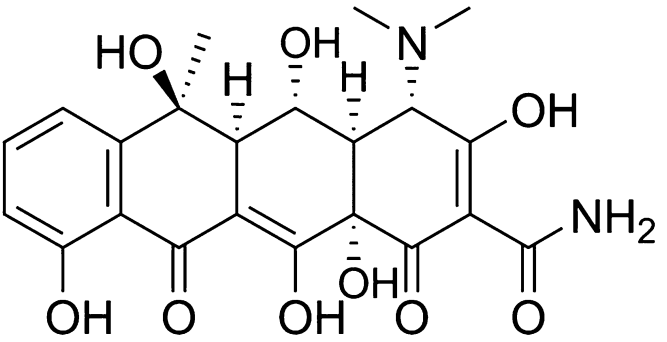

Figure 2. Molecular structure of oxytetracycline (Pickens, 2010)

With both antibiotics and pathogens taken up by crops there exists a possibility that the antibiotic inside the plants can sustain and affect the resistance level of the human pathogens internalized in the plants through a mechanism called cross resistance.

Approximately 42,000 cases of *Salmonella* contamination of food crops are reported per year in the US with 400 of them resulting in death (Center of Disease Control, 2012). *Salmonella* is best known as a pathogen found in poultry and eggs, but currently more outbreaks are caused by contaminated produce (Ayers, 2009). *Salmonella* causes those infected to develop diarrhea, fever, and cramps for 4-7 days. Most people infected are either infants, the elderly, or those with impaired immune systems (Center of Disease Control, 2012). *S.* Infantis is known to develop antibiotic resistance (Gal-Mor, 2010). In normal environments, *Salmonella* carries genes for antibiotic resistance, but they are only expressed under certain pressures (Gal-Mor, 2010).

Oxytetracycline (OTC) (**Figure 2**) is a very commonly used antibiotic that works by inhibiting the bacteria's synthesis of certain proteins (Li, 2011). OTC can be prescribed to humans, but is more commonly used in animal agriculture via concentrated animal feeding operations (CAFOs) in order to prevent diseases in livestock (Perry, 2010). When used in livestock it is commonly prescribed at the sub-therapeutic level. However, OTC does not always get fully digested and is often excreted through urine or feces into the environment and is later frequently detected in treated wastewater, surface water, and groundwater (Li, 2011).

3. Materials

Oxytetracycline (OTC) was purchased from MP Biomedicals, Solon, OH and stored at 4°C. *S.* Infantis was grown to a concentration of 10^9 CFU/mL. Lettuce (*Lactuca sativa*) cultivar Green Salad Bowl was purchased from Peaceful Valley Farm & Garden Supply, Grass Valley, CA. Seeds were stored at 4°C. Hoagland Basal Salt was purchased from MP Biomedicals, Solon, OH and stored at room temperature and autoclaved after dissolving in sterilized water. Luria Broth (LB) was made by dissolving 10g tryptone, 5g yeast extract and 10g NaCl in 1L water and was autoclaved afterwards**.**

XLD agar was purchased from BD Diagnostics Solon, OH and stored at room temperature. OTC was added until the XLD agar solution cooled down to 55°C. Phosphate buffered saline (PBS) was made by dissolving 80g of NaCl, 2.0g of KCl, 14.4g of Na_2HPO_4 and 2.4g of KH_2PO_4 into sterilized water. PBS mixture was sterilized by autoclaving and stored at room temperature.

4. Methods

4.1 Planting

The experiment was conducted using the lettuce (*Lactuca sativa*) cultivar Green Salad Bowl , the antibiotic oxytetracycline (OTC) and the model human pathogen *Salmonella enterica* serovar *Infantis* (*S.* Infantis). Initially, thirty-six 200-mL glass jars were used to grow the lettuce hydroponically. Small Styrofoam disks with holes punched out of the in center and filled with cotton were used to provide support for lettuce growth in the liquid medium. 50 mL of Hoagland Basal salt mixture was added to each jar and used as a source of nutrients. Before seeding, lettuce seeds were sterilized with 1% sodium hypochloride for 1 minute and then rinsed with sterile water three times each (Klerks et al., 2007). Three lettuce seeds were placed on the cotton filled centers of the Styrofoam disks and set in the jars. Each jar was covered with Parafilm covering to avoid contamination from microbes in the air. Plants were then left to grow in a green house until harvest time. Lettuce plants were thinned from 3 to 1 plant per jar after the first week.

4.2 Lettuce Harvests

Lettuce was harvested at 21, 35, and 48 days after planting. At each date, one third of the plants were destructively harvested. Five days before each harvesting the plants were moved to an area with representative artificial lighting where *Salmonella* and OTC were added. One day before inoculation, 100 µL *Salmonella* stock solution was added to 100mL Luria Broth (LB) nutrient solution and incubated at 37°C for 18-20 hours with shaking. After incubation, 50 mL of *Salmonella* culture was centrifuged to separate the broth and the bacteria. The supernatant was discarded and 5 mL phosphate buffered saline (PBS) solution was added to the pellet to make a concentration of 10^10 CFU/mL. 100 µL of *Salmonella* solution was added to all jars. OTC was dissolved into methanol at a 0.1 g/ 10 mL ratio. The OTC mixture was sterilized by a filtration through a 0.20 µm Polytetrafluoroethylene (PTFE) membrane filter (Cole-Palmer, Vernon Hills, IL) and diluted with water tenfold. 200 µL of OTC solution was added to half of the jars.

4.3 Addition of Oxytetracycline

OTC was added into the dissolved XLD agar growth medium in the concentrations of 0, 1, 2, 4, 8, 16, or 32 mg OTC/L of XLD. Twenty mL of each concentration of OTC agar solution was poured onto petri dishes and left to solidify. Each plant was cut at the cotyledonary node and its mass weighed (Dong et al., 2003). They were then individually washed with sterilized water. Samples were placed individually in bags and finely blended with mortar and pestle. Two mL PBS buffer was added to the ground lettuce sample (Dong et al., 2003). One mL of undiluted PBS plant solution was added to two test tubes. A third test tube had been the homogenate diluted 10 fold with PBS and a fourth test tube had the homogenate diluted 100 fold with PBS. One day before harvest, a control lettuce sample without OTC was prepared with a *Salmonella* stock solution (10^9 CFU/mL). One hundred µL of stock solution was added to 100 mL Luria Broth (LB) nutrient solution and left to mix and incubate 37°C for 18-20 hours. This was then diluted with PBS to concentrations of 10^5, 10^4, 10^3, 10^2 CFU/mL. A multichannel pipette was used to distribute 10 µL each dilution onto the agar plates in duplicates using a 4×4 drop plate method (modified from [Chen et al., 2003]). After incubating for 16-18 hours, colonies were counted. This information was used to determine the response profile to *Salmonella* of oxytetracycline.

5. Results

5.1 Harvest 1

The first harvest occurred 21 days after planting. At this time, traces of algal contamination were found in many of the lettuce jars. It later went on to kill many of the plants, which reduced the planned sample size from 12 lettuce plants per harvest to the number of surviving plants for each condition. Lettuce samples that did not show severe algal growth were harvested and tested. In the first harvest, 4 lettuce plants receiving OTC and 3 lettuce plants not receiving OTC were harvested, with 2 and 1 plants in each group having mild algal growth. Due to the highly varied concentrations of bacteria in each plate, the results were presented as normalized results. Data displayed on graphs represent the averages from samples tested each harvest. There was a decrease in normalized *Salmonella* concentration between XLD plates containing 16 and 32 mg/L OTC for all samples **(Figure 3)**. *Salmonella* grown in lettuce receiving OTC showed a lower MIC than *Salmonella* grown in LB medium and *Salmonella* grown in lettuce not receiving OTC.

5.2 Harvest 2

The second harvest occurred 35 days after planting. Three samples of *Salmonella* grown lettuce receiving OTC and 3 samples of *Salmonella* grown in lettuce not receiving OTC were used. Jars with mild algal growth were not excluded from this harvest. The colony count of *Salmonella* in agar plates amended with 0 to 8 mg/L OTC fluctuated little **(Figure 4)**. From 8 to 16 mg/L OTC, the colony count of *Salmonella* grown in LB media dropped to 0. The concentration of *Salmonella* grown in lettuce not receiving OTC decreased gradually from 8 to 32 mg/L OTC. *Salmonella* grown in lettuce receiving OTC showed resistance to the antibiotic until the concentration reached 32 mg/L OTC.

5.3 Harvest 3

The third harvest occurred 48 days after planting. Two samples of *Salmonella* grown lettuce receiving OTC and 2 samples of *Salmonella* grown in lettuce not receiving OTC were used. Each condition had 1 jar with algal contamination. From 0 to 4 mg/L OTC all plates showed little change in *Salmonella* concentration **(Figure 5)**. From 4 to 8 mg/L OTC all plates

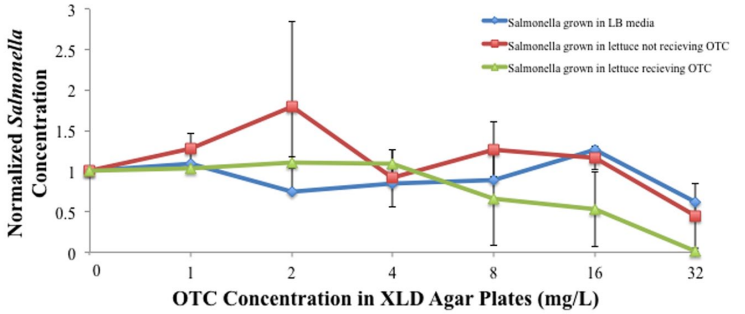

Figure 3. Graph of results from harvest 1 showing relationship between antibiotic concentration and *Salmonella* concentration in each of three growth conditions.

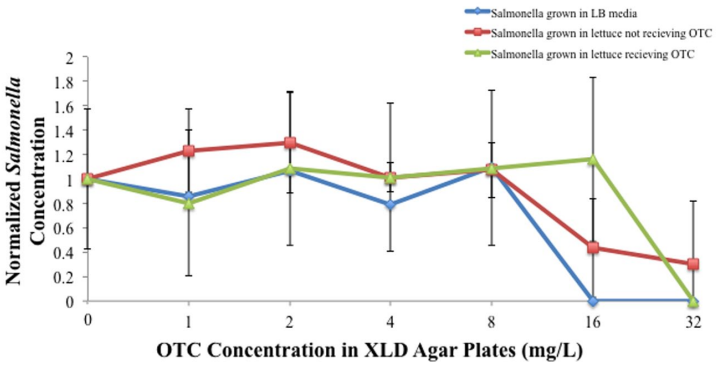

Figure 4. Graph of results from harvest 2 showing relationship between antibiotic concentration and *Salmonella* concentration in each of three growth conditions.

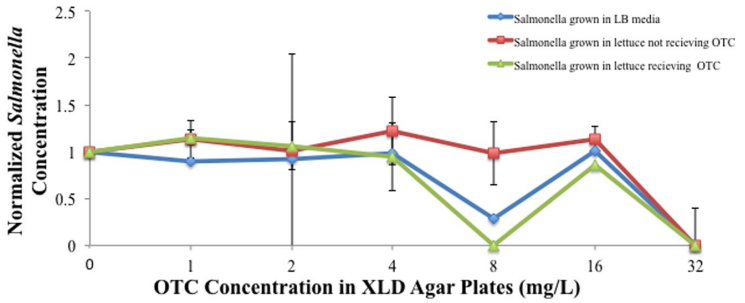

Figure 5. Graph of results from harvest 3 showing relationship between antibiotic concentration and *Salmonella* concentration in each of three growth conditions.

showed a decrease in normalized *Salmonella* concentration. *Salmonella* concentration rebounded at 16 mg/L OTC in all plates. Growth was completely inhibited in all plates at 32 mg/L OTC.

5.4 All Harvests averaged

Comparing the averages of all harvests, the proportion of *Salmonella* grown in lettuce not receiving OTC showed the most resistance to OTC overall **(Figure 6)**. The *Salmonella* grown in lettuce receiving OTC showed similar patterns compared to *Salmonella* grown in LB medium until the concentration changed to 32 mg/L OTC. All of the samples showed a decrease in *Salmonella* growth at 32 mg/L OTC. A total of 8 *Salmonella* samples grown in lettuce receiving no OTC, 9 *Salmonella* samples grown in lettuce receiving no OTC, and three samples of S*almonella* grown in LB medium were used in this experiment.

5.5 Harvest

The graph of *Salmonella* grown in lettuce receiving OTC for all harvests was used to find relationships between plant maturity and resistance levels of endophytic *Salmonella* **(Figure 7)**. In harvest 1, the *Salmonella* concentration remained steady from 0 to 4 mg/L OTC and gradually decreased to 0 from 8 to 32 mg/L OTC. In harvest 2, *Salmonella* showed

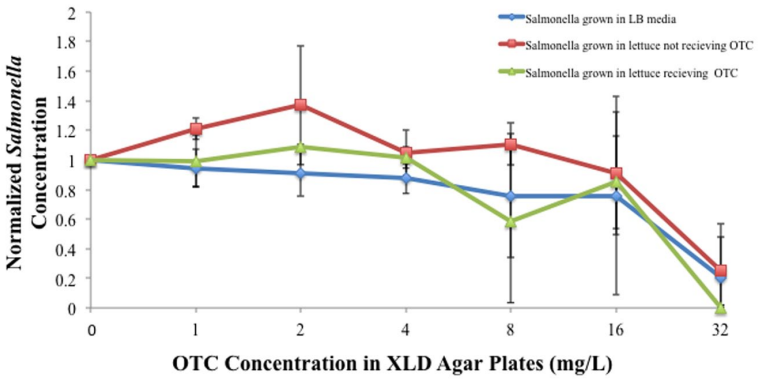

Figure 6. Graph of results from all harvests averaged showing relationship between antibiotic concentration and *Salmonella* concentration in each of three growth conditions.

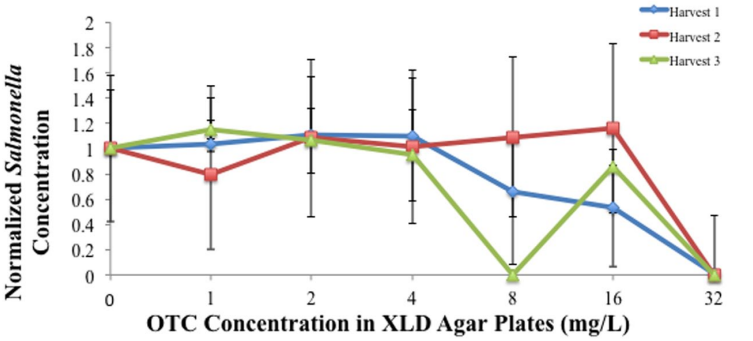

Figure 7. Graph of results from *Salmonella* growth from each harvest showing relationship between plant maturity and antibiotic resistance.

resistance to the antibiotic until the concentration reached 32 mg/L OTC. In harvest 3, little changed in *Salmonella* concentration was shown from 0 to 4 mg/L OTC. At 8 mg/L OTC harvest 3 showed a decrease in normalized *Salmonella* concentration, but it rebounded at 16 mg/L OTC and was completely inhibited at 32 mg/L OTC.

6. Discussion

Though Parafilm lids, autoclaving, and personal protection equipment was used to prevent contamination, approximately half of the plants became contaminated with algae. This was noticed at the second week of the experiment. Each week a few of the lettuce plants in the contaminated jars were dead due to algae, making it impossible to study 12 plants per harvest as originally planned. Water was replaced in all jars after noticing the contamination in hopes of removing the algae.

Neither the exposure of *Salmonella* to OTC or the influence of lettuce maturity impacted resistance levels to OTC. It was found that the conditions in which the *Salmonella* was grown had little impacts on the growth of *Salmonella* on XLD agar plants amended with various concentrations of OTC. The samples of *Salmonella* grown in LB media, lettuce receiving OTC, and lettuce receiving no OTC showed very similar antibiotic resistance levels. This was consistent in all harvests. The maturity of the plants did not significantly impact the resistance level to OTC. The differences between harvest times were not significant. This was observed in all growth conditions. The resistance level of antibiotic *Salmonella* was determined to be between 16 and 32 mg/L OTC. The averages of ev-

ery sample indicated that 32 mg/L OTC was inhibitive to all *Salmonella* samples. In future experiments, a more exact threshold could be found. The scope of the experiment could be widened. Other vegetables often eaten raw, such as tomatoes or spinach, could be used, as could other commonly used antibiotics such as amoxicillin or tetracycline. More traditional soil growth methods could be used instead of hydroponics in order to simulate large scale production conditions.

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