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MICROBIAL SOLUTIONS

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Case Study: Would you like *E. coli* with that?

Industrial microbiologists play a key role in ensuring manufacturing operations are under a state of control and end products are safe and effective. However, production facilities can still suffer from microbial contamination. In those cases, it becomes imperative to quickly find the source of the contamination and eradicate it before (more) product is impacted and scrapped. Using Charles River's AccuGENX-ST®, a cGMP service for single- or multi-locus sequence typing, it is possible to rapidly and reliably track and trend the microorganisms encountered in aseptic and non-sterile production facilities. Here, a case study is presented where AccuGENX-ST® was used to facilitate a contamination investigation.

Background

Company A is a contract dietary supplement and beverage manufacturing company. All incoming raw ingredients and all finished product lots are tested and released based on the presence/absence of *E. coli, S. aureus,* and *Salmonella* per USP <2022> *Microbiological Procedures for Absence of Specified Microorganisms – Nutritional and Dietary Supplements*. While *Salmonella* and *S. aureus* are rarely recovered, an *E. coli* positive ingredient is typically obtained about 2 times a year (usually in a botanical ingredient), which is then rejected and returned to the supplier. A finished product has never tested positive for *E. coli* until now.

Company A recently started manufacturing several powdered beverage products for a new client. One of those products has had 12 lots manufactured over the last 10 months, and 5 of the 12 finished lots have tested positive for *E. coli*. None of the raw ingredients that went into those 5 lots tested positive for *E. coli*. Manufacturing occurs on shared equipment and none of the other products tested positive for *E. coli*. Increased environmental monitoring and sampling did not reveal any sources of *E. coli*.

Methods

Release testing of raw ingredients and final product for the presence/absence of *E. coli* occurs by adding 10 grams of a single ingredient or final product into 90 mL of tryptic soy broth (TSB). The media is incubated for 24 hours and plated onto MacConkey agar. Any colony that displays characteristics of *E. coli* is identified by DNA sequencing.

Microbial identification by 16S DNA sequencing is a robust technology, but only provides resolution to the species level. Increased discrimination to the strain level can be achieved with AccuGENX-ST®, a microbial characterization process used to distinguish closely related microorganisms by utilizing well-established highly accurate sequencing methods, based on single- and multi-locus sequence typing (S/MLST).

The principle of S/MLST is simple: the technique involves PCR amplification of single or multiple gene loci (regions) that show higher variability within a species followed by DNA sequencing. These regions include essential outer membrane protein coding genes or housekeeping genes that encode for proteins necessary for normal cellular functions of the organism, all of which contain more variability in their sequence than 16S. Nucleotide differences between strains are subjected to comparative DNA sequence analysis. All the sequences from each gene target are aligned and compared to the other organisms' sequence data and the level of divergence or conservation

between the organisms is calculated and displayed with a phylogenetic tree. The data is interpreted, and a report generated to state whether the isolates are different or indistinguishable from each other based on the genes sequenced.

Results

One isolate from five different contaminated lots of final product were sent to Charles River for strain typing using the AccuGENX-XGST® service. AccuGENX-XGST® is a specialized subset of AccuGENX-ST® that uses a well-established 7 gene MLST for tracking sequence types. The *Escherichia coli* identification was confirmed for all five isolates by DNA sequencing. Each isolate was then sequenced for the adk, fumC, gyrB, icd, mdh, purA, and recA genes. The MLST analysis of the five *E. coli* strains isolated from the final product lots showed five different sequence types, indicating multiple strains of *E. coli* are involved in the final product contamination. The ATCC Control strain from the QC laboratory was also sequenced and compared to ensure that the contamination did not originate from the laboratory.

Figure 1. Phylogenetic trees of Final Product and ATCC Control strain

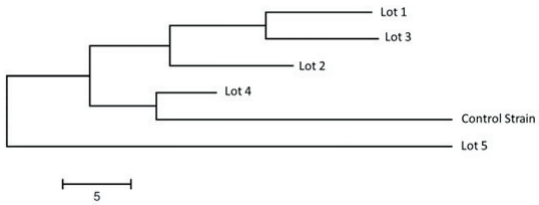

Table 1. Sequence Types

Sample	Sequence Type
Lot 1	847
Lot 2	2016
Lot 3	8823
Lot 4	206
Lot 5	720
ATCC Control Strain	3021

The final product formulation consists of 10 botanical ingredients. However, routine compendial testing didn't recover any *E. coli* positive plates from ingredients that were individually tested. It is important to note here that a number of those ingredients are spices, and research has indicated that they can have anti-microbial properties. It was hypothesized that the anti-microbial properties of the spices were inhibiting recovery of *E. coli* while testing the raw ingredients. To overcome this, method suitability studies were run to determine the appropriate dilution for each of the raw materials and final product. Increasing volumes of TSB and TSB with tween and lecithin were tested while maintaining the 10 gram volume of material. A control strain of *E. coli* was spiked into each dilution and recovery assessed. Results were read at both 24 and 72 hours, and a hit at either time point indicated a positive result.

The study found that the appropriate dilution varied for each of the raw materials. See Table 2 for optimized test method information. The raw ingredients were then re-tested for *E. coli* following the new procedure. All recovered suspected *E. coli* colonies were submitted to Charles River for microbial identifications and strain typing.

Table 2. Re-testing of raw ingredients using optimized test method and outcome

Ingredient	Optimized Test Method	Optimized Test Method
Ingredient 1	10 g into 1 L TSB + tween + lecithin	Present*
Ingredient 2	10 g into 600 mL TSB + tween + lecithin	Present
Ingredient 3	10 g into 700 mL TSB	Absent
Ingredient 4	10 g into 300 mL TSB	Absent
Ingredient 5	10 g into 90 mL TSB	Present
Ingredient 6	10 g into 90 mL TSB	Absent
Ingredient 7	10 g into 1 L TSB or 1 L TSB + tween + lecithin	Absent
Ingredient 8	10 g into 200 mL TSB	Absent
Ingredient 9	10 g into 90 mL TSB	Absent
Ingredient 10	10 g into 300 mL TSB	Absent

*Ingredient 1 was found to be accidentally contaminated with the QC strain of *E. coli*. Upon retesting, Ingredient 1 was negative for *E. coli*.

Figure 1. Phylogenetic trees of final product lots and raw ingredients

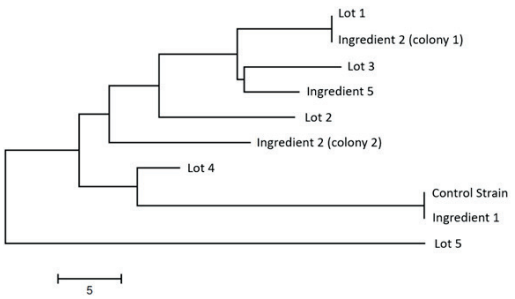

Table 3. Sequence types of final product lots and raw ingredients

Sample	Sequence Type
Lot 1	847
Lot 2	2016
Lot 3	8823
Lot 4	206
Lot 5	720
Control Strain	3021
Ingredient 1	3021
Ingredient 2 (colony 1)	847
Ingredient 2 (colony 2)	644
Ingredient 5	2607

The results from raw ingredient re-testing showed Ingredient 2 and Ingredient 5 were positive for *E. coli*. The recovered isolates were further tested using the AccuGENX-XGST® service. The MLST evaluation showed that one of the isolates recovered from Ingredient 2 showed same sequence type as one of the contaminated final product lots. However, the isolates recovered from Ingredient 5 and "colony 2" from Ingredient 2 are new strains of *E. coli,* not previously reported from final product.

Ingredient 1 also tested positive for *E. coli* and matched the sequence type of the ATCC Control strain. It was concluded that this was a sampling error from the method suitability study and not the actual raw ingredient.

Discussion

The AccuGENX-XGST® strain typing results indicated that each contaminated lot was a different sequence type. This was surprising as each sequence type is assumed to indicate a different contamination source so this would imply that each contamination event was an independent and unique event. The probability of this is very low and adds complexity in finding the source.

However, a close inspection of the raw ingredients' recA and icd sequence data by Charles River's Research & Development team, which consisted of two separate amplifications to increase sensitivity, revealed mixed electropherogram peaks for three of the ingredients. This indicates that instead of a single *E. coli* strain being present, there's really a population of Enterobacteriaceae microorganisms that also include multiple strains of *E. coli*. The final product lots are potentially contaminated by multiple ingredients that are again contaminated by more than one *E. coli* strain. The results from the PCR screens agreed with the results from the optimized testing procedure, where two of the ingredients were positive for *E. coli* and the third positive for an Enterobacteriaceae organism. Contamination of the raw ingredients is logical as most of the ingredients are natural botanicals that exist in heavily contaminated environments, such as soil fertilized with manure.

After the method suitability tests were completed, the raw ingredients were re-tested for *E. coli*. As previously mentioned, three raw ingredients were found to be contaminated. The MLST analysis of *E. coli* strains isolated from Ingredient 2 following the optimized testing procedure had at least one identical sequence type as the recovered *E. coli* strain of one of the final product lots. This confirms that Ingredient 2 was the source of at least one contaminated final product lot. It's likely that the other sequence types don't match because the contaminant *E. coli* colony was not selected for testing from the populations of *E. coli* in the contaminated raw ingredients.

While the typical paradigm is that one sequence type equals one contamination source, this case study proved the need to scientifically consider the nature of raw ingredients. The typical paradigm may apply to chemically synthesized materials, but it is unrealistic in the case of these raw botanicals that grow in high microbial load environments.

Moreover, it was critical to perform a method suitability test even though this is not required by USP for nutritional and dietary supplementals because the ingredients are natural botanicals with anti-microbial properties. By undertaking the method suitability studies, an optimized procedure was developed for each raw ingredient that can detect low levels of *E. coli* in both the raw ingredients and the final product. These test methods are thus a better screen for ingredients which will hopefully prevent future contamination events.

All 10 ingredients that are used in the final product are sourced from specific suppliers. Three are unique ingredients used only in this final product. However, the other seven ingredients are used in other products but are sourced from different suppliers. Those final product lots tested negative for *E. coli*. Is the contamination of those ingredients specific to the supplier or was the bioburden not detected with the original, less-sensitive test method? Repeat testing with the optimized method is necessary to determine if there is a difference in raw ingredient quality from different suppliers or if the bioburden is inherent in these botanicals.

Summary

Re-occurring *E. coli* contaminations of a single final product spurred a comprehensive investigation at Company A. No recovery of *E. coli* during increased environmental monitoring prompted the investigators to reconsider the raw materials, which initially tested negative for *E. coli*. After the test method was optimized for each ingredient, they were re-tested for the presence/absence of *E. coli*. All suspicious colonies were identified by Charles River and AccuGENX-XGST® strain typing was performed on all confirmed *E. coli* isolates. Two raw ingredients were confirmed to be contaminated with multiple strains of *E. coli*, which explain the various sequence types determined in each of the contaminated final product lots.

Genetic analysis and AccuGENX-XGST® strain typing played a critical role in discovering the source of contamination. Without a thorough investigation and true root cause, manufacturing operations and final product is still in danger of future contamination events. This could result in manufacturing delays, drug shortages, product recalls, increased costs, harm to the brand, and increased regulatory scrutiny. With all that at risk, are you doing everything you can to get to root cause when contamination occurs?

Learn more about strain typing services at www.criver.com/accugenix-strain-typing.

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askcharlesriver@crl.com • www.criver.com