Real-time PCR detection of *Staphylococcus aureus* in pharmaceutical products contaminated with mixed bacterial cultures

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The contamination of pharmaceuticals by microorganisms represents a major threat to public health, not just in the USA and Europe, but all around the world. Pathogenic microorganisms cause morbidity and in some cases mortality when present in products.[1,2] Spoilage due to microbial breakdown of formulations can affect potency and shelf life, yet non-sterile pharmaceuticals contain a microbial bioburden that is not detrimental to the formulation or hazardous to consumers.

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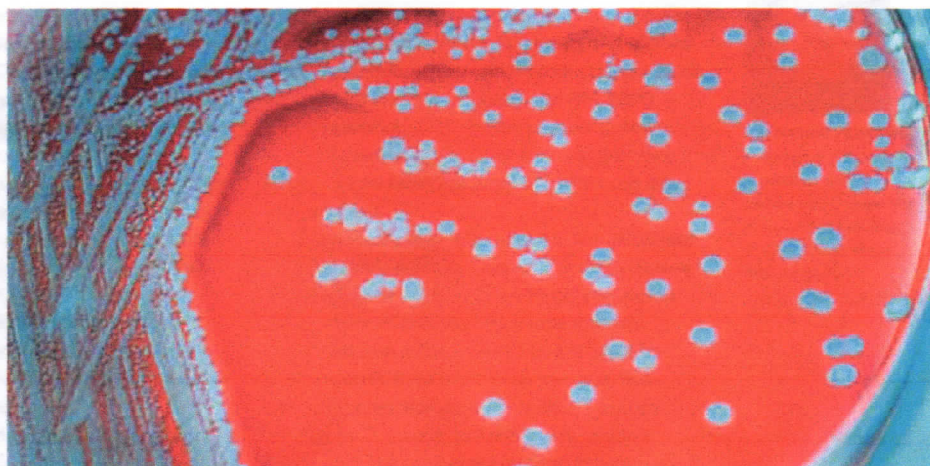
BASED UPON compendial tests, when non-sterile pharmaceuticals are tested, objectionable microorganisms must be absent from the finished product and raw materials.[1,2] For this reason, regulatory agencies around the world require the absence of certain types of microorganisms that can be considered hazardous to consumers or compromise drug stability and efficacy. *Staphylococcus aureus* is one of the indicator microorganisms that must be absent from non-sterile pharmaceutical drugs; however, based upon published scientific surveys, *S. aureus* is a frequent microbial contaminant in both sterile and non-sterile pharmaceutical products around the world.[1-3] Furthermore, infections by *S. aureus* are the number one cause of nosocomial outbreaks in the USA,[4] while common infections by *S. aureus* are related to skin eruptions, bacteremia, endocarditis, toxic shock syndrome and pneumonia.[5] *S. aureus* is also characterised by the ability to develop resistance to almost any antibiotic.

The challenge for pharmaceutical manufacturers is to produce a non-sterile drug with low levels of microorganisms and a complete absence of the 'wrong type' of objectionable microbes.

Some guidance to determine what is objectionable is provided by the Code of Federal Regulations and the USP informational chapter <1111>.[2] Most pharmaceutical companies have relied on traditional cultivation and phenotypic methods to isolate and identify microbial contaminants;[1,6] however, these methods are time consuming (5–7 days) and laborious, and rely upon the growth of microorganisms on specific substrates in the media. Of note, some bacteria do not grow on those substrates or otherwise grow too slowly to be detected by the incubation times currently used. In some cases, microbial cells undergo a physiological state by reducing metabolic reaction rates and cell size, which affect the protein and enzymatic profiles used to identify environmental isolates.[6] These microbial cell changes are triggered by physical processes and environmental systems implemented to reduce or eliminate microorganisms during pharmaceutical manufacturing. If the processes and systems are not validated or properly implemented, microorganisms can contaminate products, raw materials and equipment.[1,2]

Conventional methods relying on selective agar media after enrichment in tryptic soy broth (TSB) containing 2 percent Tween 20 were used to detect S. aureus in pharmaceutical samples with low levels of bacterial contamination – eg, below 75 colony-forming units (CFU). The selected bacteria were commonly found in products recalled in the USA.[1,2] Following inoculation, samples were shaken for 18–24 hours at 37°C to promote bacterial growth. *Table 1* shows the results for each selective agar media analysed: phenylethyl alcohol (PEA) and mannitol salt agar (MSA). All seven products tested were found to show bacterial growth. Identification of colonies growing on selective agar media for Gram-positive bacteria, PEA and MSA, showed S. aureus. However, the antiseptic mouthwash did not show any bacterial growth on PEA but S. aureus was isolated and identified on MSA.

Bacterial DNA was then extracted from all contaminated products. To determine the predominant bacterial DNA in the extracted product suspensions, a regular PCR reaction was performed targeting the 1.5 kilobases (kb) 16S rRNA eubacterial gene – a universal gene present in all members of the domain bacteria.[7] The gene has conserved and variable regions that can be used to distinguish bacterial phyla, genera and species. The amplified fragments from product suspensions were cloned into vector plasmid pCR®4-TOPO. DNA sequencing of the clones demonstrated the predominant presence of E. coli in the clone library, but no other bacterial 16S rRNA sequence was detected.
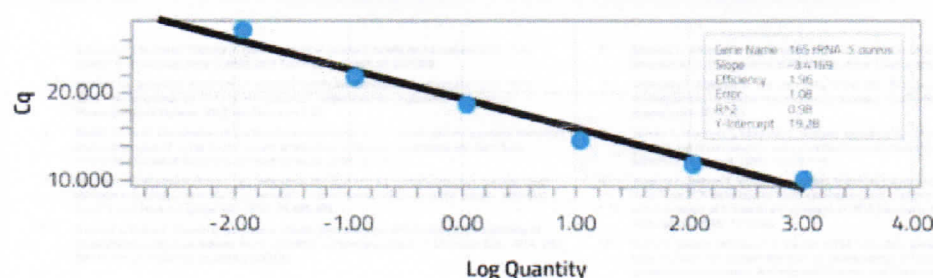
To specifically detect S. aureus in the extracted bacterial DNA, a 273bp fragment of the S. aureus 16S rRNA gene was used as a target to develop the real-time PCR assay.[8] Real-time PCR was performed with a LightCycler System[9] using SYBR green PCR master mix as the detection system in a reaction mixture of 25ul. The copy number for S. aureus 16S rRNA gene is either five or six.[10] The DNA concentration for undiluted

TABLE 1 Isolation and identification of bacterial contamination on selective media from pharmaceutical products contaminated with mixed bacterial cultures and real-time PCR detection

Sample	PEA	MSA	RT-PCR Average quantification cycle (Cq)
Cough suppressant liquid	+ S. aureus	+ S. aureus	24.66
Antiseptic mouthwash	–	+ S. aureus	22.59
Itch stopping cream	+ S. aureus	+ S. aureus	22.33
Calamine lotion	+ S. aureus	+ S. aureus	20.91
Liquid insect repellent	+ S. aureus	+ S. aureus	21.42
Hydrocodone Acetaminophen tablets	+ S. aureus	+ S. aureus	16.80
Acetaminophen tablets	+ S. aureus	+ S. aureus	26.29

+ = growth
– = no growth

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FIGURE 1

Gene Name	16S rRNA S. aureus
Slope	-3.4169
Efficiency	1.96
Error	1.08
R^2	0.98
Y-Intercept	19.28

Cq / Log Quantity

LEFT: Standard curve analysis of S. aureus DNA dilutions after real-time PCR

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samples of *S. aureus* DNA was found to have 1,144 nanograms (ng) per ml with a Cq of 10.13 (*Table 2*). The Cq indicated the cycle at which fluorescence from amplification exceeded the background fluorescence. When 10-fold dilutions of *S. aureus* DNA were tested, the minimum concentration detected was 0.01144ng/ml with a correlation coefficient of 0.98 (*Figure 1*). The Cq for the lowest DNA concentration detected was 27.04. Melting curve analysis of the amplified fragments confirmed the presence of the 273bp fragment.

All contaminated products analysed were found to have Cq values ranging from 16.80 to 26.29 (*Table 1*). The faster Cq values were found with

the Hydrocodone Acetaminophen tablets, while Acetaminophen tablets exhibited for the longest amount of time. This indicated that the growth conditions and neutralisation protocol provided optimal conditions for the low numbers of *S. aureus* to grow sufficiently in the presence of other bacteria. *S. aureus* DNA can therefore be extracted from the bacterial mixed culture using a mild DNA extraction protocol and subsequently detected by real-time PCR. The low *S. aureus* inoculum of 24CFU/ml was easily detected after a 24-hour enrichment with shaking at 37°C.

Conclusions

A real-time PCR assay was developed to detect *S. aureus* in pharmaceutical products contaminated with low numbers of bacterial contamination by *Burkholderia cepacia, Escherichia coli, S. aureus,* and *Bacillus megaterium*. All samples showing positive results with standard microbiological methods were also positive with real-time PCR. Rapid assessment of pharmaceutical samples provides important information for quality control and highlights the possible risk of contaminated material, allowing the expeditious implementation of corrective actions to prevent morbidity and mortality by the lack of process control during manufacturing of pharmaceutical products.

📊 **TABLE 2** **Real-time PCR detection of *S. aureus* DNA dilutions**

[DNA] ng/ml	Quantification Cycle (Cq)
1144	10.13
114.4	11.52
11.44	14.83
1.144	18.82
0.1144	21.87
0.01144	27.04

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