UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

Utilização da tecnologia de MALDI-TOF para identificação acurada de bactérias obtidas de placas de monitoramento de áreas limpas de uma indústria de medicamentos estéreis.

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Porto Alegre

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Trabalho de conclusão de curso apresentado por Bruna Aguiar Thomas como requisito parcial para obtenção de GRAU DE FARMACÊUTICO.

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Título: Utilização da tecnologia de MALDI-TOF para identificação acurada de bactérias obtidas de placas de monitoramento de áreas limpas de uma indústria de medicamentos estéreis.

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RESUMO

Métodos rápidos para identificação microbiana aplicados ao monitoramento ambiental de áreas limpas de indústria de medicamentos são fundamentais e garantem a qualidade do produto, bem como, a segurança do paciente. Sendo assim, o objetivo deste trabalho foi identificar todas as colônias de bactérias isoladas em placas de monitoramento alocadas nas quatro áreas limpas de uma indústria de medicamentos estéreis. Foram submetidas à identificação no sistema de MALDI-TOF Microflex LT® todas as unidades formadoras de colônias (UFC), independentemente do número presente nas placas de monitoramento, no período de setembro a novembro de 2019. Das 90 UFC obtidas, 92,2% (83/90) foram identificadas, sendo que 79 colônias apresentaram identificação confiável à nível de espécie. As espécies com maior frequência foram: *Micrococcus luteus* (55,42%), *Staphylococcus epidermidis* (14,46%) *e Staphylococcus simulans* (6,02%). O maior número de colônias foi proveniente das salas de Grau C e D sendo que não foram obtidas colônias nas placas das salas de Grau B. Nas placas alocadas em sala de Grau A foram observadas apenas 2 UFC, sendo elas identificadas como *Paenibacillus naphthalenovorans* e *Staphylococcus epidermidis*. O monitoramento das áreas limpas de uma indústria é mais baseado em aspectos quantitativos, mas a identificação precisa dos microrganismos eventualmente encontrados apresenta-se como um diferencial importante para o melhor gerenciamento da higienização das salas.

Palavras-Chave: Identificação microbiana, Áreas limpas, MALDI-TOF, medicamentos estéreis.

INTRODUÇÃO

Áreas limpas são ambientes nos quais a concentração de partículas em suspensão no ar é controlada sendo que estas áreas são construídas e utilizadas de forma a minimizar a introdução, geração e retenção de partículas em seu interior [1,4,5,7]. Para cumprir com os requisitos de Boas Práticas de Fabricação é fundamental que as áreas destinadas a processos assépticos possuam um qualificado programa de limpeza e um apropriado sistema de tratamento de ar. Este sistema deve ser capaz de oferecer proteção ao produto durante as etapas da produção e proteger o meio ambiente de contaminantes provenientes do processo fabril [4,7].

As áreas limpas são classificadas segundo condições ambientais conforme a quantidade de partículas viáveis e não viáveis em dois diferentes momentos de realização dos testes - "em operação" e "em repouso" [4,5]. As resoluções publicadas pela Agência Nacional de Vigilância Sanitária (ANVISA), oriundas de documentos técnicos da Organização Mundial da Saúde (OMS) recomendam a distribuição das salas de produção em quatro graus A, B, C e D. Esses graus são diferenciados de acordo com a atividade executada e, também, pelas características exigidas do ambiente. O Grau A define uma área crítica que é utilizada em operações de alto risco, como a zona de envase, e nessa área são colocados os reservatórios de tampas, ampolas abertas e frascos-ampola. A área que circunda a área de Grau A é denominada área de Grau B, a qual, também, é considerada uma área crítica. As áreas consideradas menos críticas, como salas de manipulação e paramentação são denominadas áreas de Grau C e D [8].

Devido à possibilidade de contaminação microbiana durante a produção de medicamentos estéreis, o monitoramento microbiológico das áreas é uma importante ferramenta, já que a presença de microrganismos pode afetar diretamente a segurança do

paciente e a qualidade do produto [4,7]. Portanto, é de responsabilidade da indústria planejar, elaborar, implementar e documentar o monitoramento, mediante análise de risco [4,5]. Os pontos de amostragem determinados no programa de monitoramento devem ser representativos de todas as áreas limpas e considerar a proximidade do produto e a possibilidade deste entrar em contato com o ar e com superfícies existentes na sala [4,5].

Os níveis de contaminação estabelecidos pela indústria baseiam-se no histórico obtido pelo monitoramento das áreas limpas e devem ser inferiores aos recomendados pela legislação vigente [3]. Quando o limite de alerta é superior às condições normais deve-se realizar um acompanhamento. Entretanto, quando o limite de ação é excedido, faz-se necessário realizar uma investigação e acompanhamento imediato, e caso necessário, a aplicação de uma ação corretiva [4,5].

A principal fonte de infecção das áreas limpas está relacionada ao operador que executará os procedimentos. Portanto, desvios das condições normais podem ser detectados, permitindo procedimentos corretivos antes que a qualidade do produto seja afetada. Métodos rápidos para identificação microbiana aplicados ao monitoramento ambiental são fundamentais e garantem a qualidade do produto, bem como, a segurança do paciente. [17]

O MALDI-TOF MS (*Matrix-Assisted Laser Desorption-Ionization Time of Flight – Mass Spetrometry)* é uma tecnologia que surgiu nos últimos anos e que permite a identificação de microrganismos de forma rápida e precisa através da técnica de espectrometria de massas que geram um perfil proteico da amostra bacteriana o qual é comparado com um banco de dados pré-estabelecido. À medida que esta técnica se torna amplamente usada, os bancos de dados ficam mais completos e melhoram ainda mais a confiabilidade na identificação [2,12].

O objetivo deste trabalho foi utilizar a metodologia de MALDI-TOF para identificar todas as colônias de microrganismos obtidas em placas de monitoramento alocadas nas quatro áreas limpas de uma indústria de medicamentos estéreis.

MATERIAIS E MÉTODOS

Escolha das áreas

O estudo foi realizado em uma indústria de fabricação de medicamentos estéreis de pequeno volume. Foram monitoradas quatro áreas limpas de graus diferentes de classificação (A, B, C e D) e em pontos diferentes, conforme legislação vigente. Estas salas foram avaliadas no período de setembro a novembro de 2019, durante a produção de medicamentos estéreis. Os locais escolhidos para o monitoramento microbiológico foram pré-definidos por uma análise de risco, totalizando 23 pontos. Em somatória a esta análise usual, semanalmente foram adicionadas quatro placas, sendo duas placas de contato e duas placas de sedimentação.

Além dos procedimentos descritos acima, foi realizado um levantamento do histórico das identificações bacterianas na indústria durante os meses de janeiro de 2018 a agosto de 2019. Esses dados históricos incluem as identificações dos microrganismos encontrados nestas áreas, apenas quando as quantificações atingiam os limites de ação estabelecidos pela legislação vigente.

Métodos de Coleta Microbiológica

As coletas foram realizadas durante a produção de medicamentos, conforme procedimentos estabelecidos pela ANVISA. Nas áreas classificadas como C e D de manipulação e paramentação, respectivamente, as amostragens foram realizadas em condição de operação. Foram utilizadas placas de Petri 90x15mm contendo Ágar TSA (*Triptone Soy Agar*) expostas por um período máximo de 4 horas. Enquanto placas RODAC® contendo MCTA (Ágar *Microbial Content Test Agar*) foram usadas para o contato, sob leve pressão, com a bancada da área circundante (B) e no visor da área de envase (A), por aproximadamente cinco segundos.

Após, as placas foram incubadas em estufa durante 48 horas a uma temperatura de 32,5°C. Aquelas que apresentaram crescimento de uma ou mais UFC, foram envoltas em papel filme, colocadas em caixas fechadas e encaminhadas para identificação no Laboratório de Pesquisa em Resistência Bacteriana (LABRESIS) do Hospital de Clínicas de Porto Alegre.

Identificação dos microrganismos

A identificação das colônias foi realizada pela tecnologia de MALDI-TOF em equipamento Microflex LT® (*Bruker Biotyper, Bruker Daltonics GmbH*, Bremen, Germany), segundo as recomendações do fabricante. As colônias foram colocadas na placa alvo do equipamento e, após fixação (secagem), submetidas a extração proteica direta com a adição de 1 µL de Ácido Fórmico a 70%. Após a total evaporação do ácido fórmico, foi colocado 1 µL da matriz HCCA (Ácido alfa-ciano-4-hidroxicinâmico) e a amostra foi submetida à identificação.

Foram consideradas satisfatórias as identificações a nível de espécie quando o escore era superior à 2.00. Quando o escore era ≤1,99 e >1,70, a identificação era

considerado aceitável a nível de gênero. Escores menores que 1.70 indicavam que não era possível identificar o microrganismo. Todas as colônias que não atingiam escore aceitável (<2.00) foram submetidas à nova captura de espectro.

RESULTADOS

Foi possível detectar crescimento de 90 UFC nas placas de sedimentação e de contato nas áreas analisadas. O crescimento das UFC foi bem variado dentre as áreas, sendo mais frequente nas placas das salas de Grau D e Grau C. Embora a positividade das placas na sala de Grau D tenha sido de apenas 50% (3/6), houve um maior número de UFC por placa nessas salas do que as de outras áreas. A área de Grau A apresentou 2,4% do crescimento total, sendo duas das placas de contato com o crescimento de 1 UFC em cada uma delas. Na área classificada de Grau B não houve crescimento bacteriano durante o período de identificação (Figura 1).

Das 90 UFC detectadas, foi possível identificar um total de 83 colônias (92,2%) pelo MALDI-TOF Microflex LT®. Destas, 79 identificadas a nível de espécie (escore >2,00) e quatro pelo menos a nível de gênero. Um total de setes colônias apresentaram escores <1.69, não sendo possível sua identificação. Do total de colônias identificadas, obteve-se a identificação de 19 gêneros diferentes e de 15 espécies. As espécies que compreenderam mais de 75% das colônias analisadas foram *Micrococcus luteus* com 55,4% (46/83), *Staphylococcus epidermidis* com 14,5% (13/83) e *Staphylococcus*

simulans com 6,0% (5/83); o percentual individual das outras espécies/gêneros foi inferior a 5% (Tabela 1).

Em paralelo a estes resultados, foi avaliado o histórico das identificações no período de janeiro de 2018 a agosto de 2019. As UFC eram identificadas quando se enquadravam em medida de alerta ou de ação, sendo assim, neste período avaliado, foram caracterizadas 47 bactérias. As áreas que apresentaram identificação de UFC foram as de Grau A com 97,9% (46/47) de prevalência e 2,1% (1/47) na de Grau B. Durante este período as demais áreas (C e D) não apresentaram crescimento de colônias acima do limite de alerta ou de ação estabelecidos. A caracterização/ identificação destas 47 UFC foi realizada por metodologia convencional (características morfológicas e provas bioquímicas). Nenhuma das 47 colônias foi identificada a nível de espécie. Dezesseis colônias foram identificadas a nível de gênero sendo o gênero *Staphylococcus* spp. (75,5% - 12/16) o mais comum. As demais colônias (30 UFC), foram identificadas apenas a nível morfológico (cocos Gram Positivos, Bacilos Gram positivos, etc...).

DISCUSSÃO

Embora índices elevados de positividade no monitoramento ambiental não sejam usuais em áreas limpas, a avaliação das placas consideradas positivas é de extrema importância para o processo fabril. No presente trabalho, foi possível observar um crescimento variado entre as áreas de Grau A, C e D. Como usualmente esperado, as placas dispostas na sala de Grau D apresentaram maior positividade que as demais, seguida da área de Grau C. Na área limpa de Grau D obtivemos o crescimento bacteriano em três das seis placas expostas neste trabalho. Embora a positividade seja de 50% (3/6) percebemos um maior número de colônias por placa foi observado quando comparadas à

de outras áreas, cerca de 67 UFC em uma das placas. Existem diferentes normas técnicas que tratam das classificações de áreas limpas, sendo as mais empregadas em território nacional as normas da ISO (*Internacional Organization for Standartization*) e ANVISA [4].

Cada etapa da operação de fabricação requer um nível de limpeza ambiental adequado, a fim de minimizar os riscos de contaminação do medicamento ou dos materiais que estão sendo trabalhados [8]. Assim como Utescher e colaboradores em 2007, estes achados demonstram que o impacto do trabalho dos operadores e seus hábitos são diretamente relacionados à contaminação bacteriana em áreas limpas, evidenciado no presente estudo os dados obtidos na sala de Grau D.

O programa de monitoramento ambiental é fundamental para a garantia da qualidade do produto fabricado, pois controla a carga microbiana viável e fornece dados dos procedimentos operacionais [17]. A maior positividade em áreas de Grau C e D é comumente esperada e ambas as salas estão relacionadas à manipulação e paramentação [17,15]. Quando se compara as bactérias da biota normal da pele humana com os microrganismos isolados nestas áreas é possível determinar uma correlação direta [15]. Com isso, pode-se afirmar que o operador tem uma contribuição com o crescimento bacteriano nas placas de monitoramento expostas nas áreas de produção.

Quanto ao crescimento bacteriano em áreas consideradas de alto risco operacional, apenas a Grau A apresentou positividade (2,4% do total de placas expostas) já nas salas limpas Grau B não foi observado crescimento. Essas são áreas críticas e de grande impacto sobre a qualidade de medicamentos estéreis e é fundamental um controle microbiológico efetivo para evidenciar situações em que onde o limite de alerta ultrapasse condições ideais de produção.

Embora não seja comum contaminações em áreas assépticas durante o monitoramento microbiológico, a presença de crescimento bacteriano não pode ser descartada. Outros autores também relataram eventuais positividades no controle desta fase da produção [9,3]. Por tratar-se de contaminações em áreas destinadas à envase de medicamentos estéreis, qualquer positividade, independente da natureza do microrganismo, pode ser prejudicial para segurança do paciente. Conforme estabelecido pela ANVISA, um lote produzido só poderá ser liberado após obtenção dos resultados do monitoramento ambiental, que comprovem que não foram excedidos os limites de ação em nenhuma das áreas críticas associadas diretamente ao preparo do produto. [4]

Ao considerar a identificação, através da tecnologia de espectroscopia de massas (MALDI-TOF), de 83 bactérias de 90 UFC nesse estudo, foi possível estabelecer um grau de assertividade maior de 90%. Assim, mesmo considerando que quatro colônias tenham sido identificadas apenas a nível de gênero e sete não tenham sido identificadas pois apresentaram valores de escore não confiáveis (<1.69), a tecnologia de MALDI-TOF pode ser considerada extremamente precisa na identificação das colônias das áreas limpas. De fato, em contrapartida às técnicas convencionais, o MALDI-TOF apresenta enormes vantagens que incluem, entre outras características alta sensibilidade e especificidade [16,13,6,3].

Cabe ressaltar que a tecnologia de MALDI-TOF permite a utilização de baixa quantidade de massa bacteriana e não requer uma incubação extra, como nos procedimentos de identificação por testes bioquímicos. Seng e colaboradores (2009) estimaram que o tempo de identificação individual de isolados por MALDI-TOF é em torno de 6 minutos, esse tempo é extremamente menor que por técnicas convencionais que variam de 6-48h. O tempo reduzido na caracterização das colônias é fundamental

para indústria de medicamentos estéreis, visto que as medidas de ação podem ser tomadas de forma mais ágil.

Ao considerar os dados do histórico das identificações prévias (janeiro de 2018 a agosto de 2019), as UFC eram identificadas apenas quando se exigiam medidas de ação, como recomendado por legislação vigente. Portanto, neste período as áreas de Grau A e B apresentaram maior número de identificação que as demais. Levando em consideração que o funcionamento correto das investigações sobre desvios nas condições normais do ambiente é um componente crítico de um sistema da qualidade [5,4,3]. É importante a utilização de métodos de identificação destas colônias mais eficazes e rápidos que os métodos convencionais utilizados.

Ao analisar as espécies mais frequentes nas áreas classificadas (A, C e D) durante o período de setembro a novembro de 2019, obteve-se a prevalência de *Micrococcus luteus*, seguido de *Staphylococcus epidermidis* e *Staphylococcus simulans*. Estes dados apresentados aqui corroboram com dados de outros trabalhos, que descreveram a elevada prevalência destas mesmas espécies [17,15,18]. Embora as áreas classificadas possuam um programa de limpeza e sanitização adequadamente validado, além de um sistema de tratamento de ar capaz de reduzir drasticamente os níveis de partículas viáveis e não viáveis, a positividade de placas de monitoramento pode ocorrer, o que indica que a ação humana nos procedimentos de preparação dos medicamentos pode ter um papel importante como agente contaminante.

A microbiota normal da pele humana é composta por um considerável número de microrganismos, que evoluíram para melhor aproveitar estas condições ambientais. A biota residente é mais limitada às espécies Gram-positivas, dentre outros microrganismos como vírus e fungos, sendo que estes agentes possuem a capacidade de tolerar o estresse

associado ao ambiente físico da pele [10]. As espécies neste trabalho relatadas, embora possam ser isoladas de amostras ambientais, são bactérias já descritas como microbiota da pele de seres humanos. A identificação de colônias de bactérias relacionadas à biota humana nestas áreas limpas evidencia que, mesmo com procedimentos adequados de paramentação e higienização das áreas, os operadores tem uma influência direta na contaminação das salas durante o processo [17]. Andrade em 2017, relatou que o mapeamento geral dos microrganismos presentes nas áreas limpas indica como mais prevalentes bactérias pertencentes ao grupo dos cocos, muito comuns na pele e na mucosa humana.

As espécies microbianas residentes são encontradas de forma consistente, embora, possa haver variação nas quantidades de pessoa para pessoa. A grande maioria dos indivíduos apresentam os gêneros *Propionibacterium, Staphylococcus, Micrococcus, Corynebacterium* e *Acinetobacter* como colonizantes [10]. Cabe ressaltar que *Micrococcus luteus* é uma das bactérias mais frequentemente detectadas em águas de sistemas industriais e, tem alta capacidade para formar biofilme, o que afeta diretamente o sistema fabril [18]. Além da capacidade desta bactéria formar biofilme, a presença em solo e pele humana justifica a alta prevalência e a identificação na sala de paramentação de operadores. Já que, a população microbiana prevalente no estudo foi proveniente das áreas classificadas Grau C e D.

Outros microrganismos prevalentes, como *Staphylococcus epidermidis* e *Staphylococcus simulans* são constantemente identificados nessas salas limpas e, também, pertencem à biota humana [11,17,3]. A identificação de diferentes espécies de *Staphylococcus* spp., revelam que operadores mesmo paramentados com macacões estéreis, necessitam também de recorrentes treinamentos para manipulação de medicamentos de maneira asséptica. Assim como Utescher e colaboradores (2007), nós

acreditamos que os resultados obtidos mostram que procedimentos operacionais podem afetar as características microbiológicas das áreas limpas.

O *Staphylococcus epidermidis* possui a capacidade de produzir macromoléculas extracelulares e de superfície celular, que podem aumentar a adesão bacteriana à corpos externos, formando eventualmente um biofilme. Essa formação é um aglomerado de células com multicamadas, cercadas por uma matriz polissacarídica extracelular o que garante a proteção a estas bactérias [14]. Apesar de sua baixa virulência, estes estafilococos são bem adaptados para aderir a superfícies metálicas e plásticas de corpos estranhos. O que os tornam um importante contaminante presente em áreas limpas.

O monitoramento das áreas limpas de uma indústria é mais baseado em aspectos quantitativos, mas a identificação precisa dos microrganismos eventualmente encontrados apresenta-se como um diferencial importante para o melhor gerenciamento da higienização das salas. Este trabalho mostrou que a identificação de bactérias por metodologias como o MALDI-TOF é importante não só quando os limites de ação são excedidos. A identificação por MALDI-TOF é rápida e confiável elevando os níveis de assertividade da identificação de bactérias. Além disso, devido às características das UFC encontradas, ficou evidenciado que o operador é a principal fonte de contaminação de áreas limpas durante o processo de fabricação.

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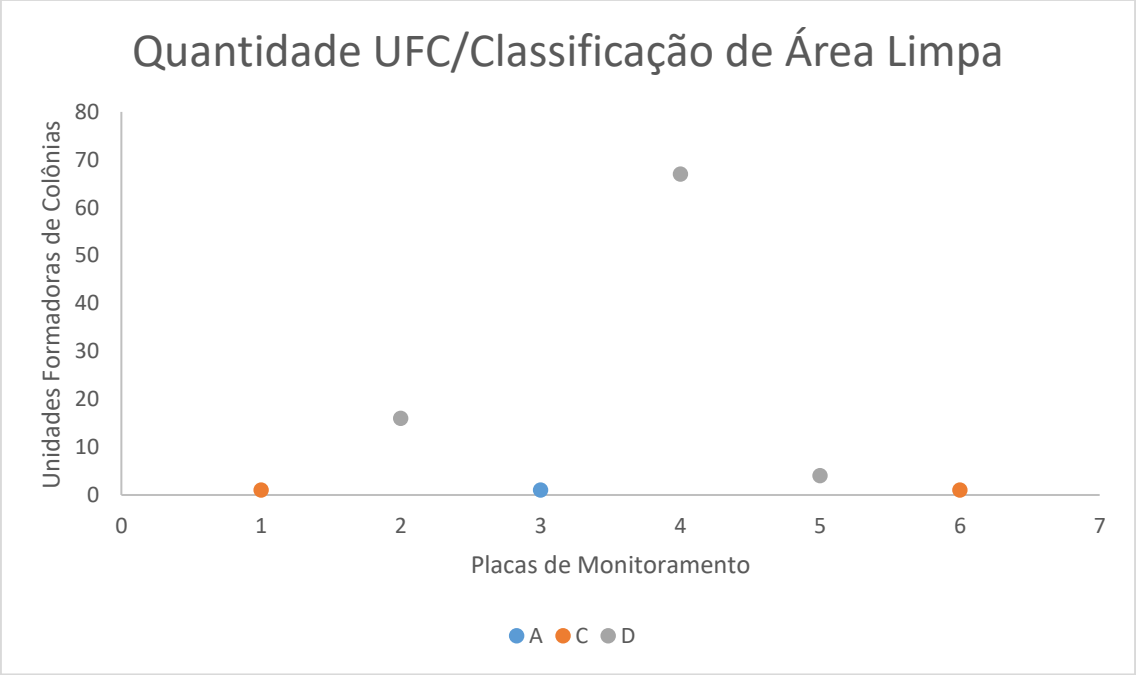
Tabela 1. Frequência de Microrganismos identificados por MALDI-TOF.

Identificação Bacteriana	**Frequência (%)**
Micrococcus luteus	55,42
Staphylococcus epidermidis	14,46
Staphylococcus simulans	6,02
Staphylococcus capitis	4,82
Moraxella sp	2,41
Paenibacillus naphthalenovorans	1,20
Brevibacterium luteolum	1,20
Staphylococcus caprae	1,20
Corynebacterium freneyi	1,20
Bacillus horneckiae	1,20
Micrococcus sp	1,20
Brevibacterium ravenspurgense	1,20
Staphylococcus haemolyticus	1,20
Staphylococcus lugdunensis	1,20
Paenibacillus thiaminolyticus	1,20
Massilia timonae	1,20
Staphylococcus hominis	1,20
Staphylococcus sp	1,20
Moraxella osloensis	1,20
Total ID	100,00

Figura 1. Gráfico de dispersão da positividade das placas de monitoramento e número de colônias nas salas limpas.



*****A sala limpa Grau B não apresentou crescimento bacteriano em nenhuma das placas de monitoramento expostas.

ANEXO I – Normas de publicação da revista "BRAZILIAN JOURNAL OF MICROBIOLOGY"

GUIDE FOR AUTHORS

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