MARIE ELIZA ZAMBERLAN DA SILVA

“COMPARAÇÃO DA QUALIDADE BACTERIOLÓGICA DE ÁGUA MINERAL, ÁGUA DE POÇOS ARTESIANOS E ÁGUA DE ABASTECIMENTO MUNICIPAL: POTENCIAL PATOGÊNICO DE *Pseudomonas aeruginosa* ISOLADAS".

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Dissertação apresentada ao Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina, como requisito parcial ao Titulo de Mestre em Microbiologia.

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Londrina, 31 de março de 2005.

DEDICATÓRIA

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LISTA DE LEGENDA

LISTA DE FIGURAS

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ANEXO

SUMÁRIO

INTRODUÇÃO

A água é o mais comum e importante composto químico na Terra. Entretanto somente ≈2.6% da água total global de $1.4x10^9$ km^3, é água doce e conseqüentemente disponível como potencial para consumo. A disponibilidade de água potável é um fator crítico para sobrevivência e desenvolvimento da vida. Na historia da humanidade, centros culturais foram sempre encontrados em áreas com suficiente abastecimento de água doce. Com o aumento da população, o abastecimento natural de água tornou-se limitado, e todas as grandes culturas desenvolveram sofisticadas técnicas e sistemas para obter acesso a novos reservatórios (ex.: perfuração de poços e construção de aquedutos) e distribuir água para irrigação e consumo (SZEWZYK, 2000). A contaminação de água natural com material fecal, esgoto doméstico e industrial entre outros, pode resultar em um aumento do risco de transmissão de doenças para o ser humano que utiliza estas águas. Doenças entéricas contraídas de água contaminada continuam sendo um sério problema em países em desenvolvimento e um menor, mas crônico, em países desenvolvidos (GRANT, 1997). Essa contaminação, que vem ocorrendo ao longo dos anos, é causada principalmente pelo desenvolvimento industrial, crescimento demográfico, e ocupação do solo de forma intensa e acelerada; isso vem provocando o comprometimento dos recursos hídricos disponíveis para consumo humano, recreação e múltiplas atividades, aumentando consideravelmente o risco de doenças de transmissão e de origem hídrica.

Segundo dados da Organização das Nações Unidas (ONU), 32 países no mundo já enfrentam escassez de água. Mais de 1 bilhão de pessoas não tem

acesso a água limpa para beber e quase 3 bilhões carecem de quaisquer serviços de saneamento público (ABINAM, 2005).

A Organização Mundial de Saúde (OMS) (2004), estima que uma pessoa em seis – mais de 1 bilhão de seres humanos – usa fontes de água potencialmente nocivas, e que aproximadamente 2,6 bilhões de pessoas estejam sem condições sanitárias. Todos os dias este desastre invisível reivindica a vida de mais de 3900 crianças com idade inferior a 5 anos. Na América Latina e Caribe, há uma população de 60 milhões sem fonte de água potável.

Nogueira *et al*. (2000), detectaram coliformes totais e fecais em 595 e 235 das amostras de água, respectivamente. Como esperado, o maior número de amostras de água contaminadas por coliformes totais (362) e fecais (159) foi encontrado em água “in natura” coletada de fontes e pontos de consumo de comunidade urbana e rural. Segundo estes autores, a maioria das pessoas de tais áreas usa a água diretamente das fontes disponíveis, sem qualquer tratamento e, portanto está exposta a uma variedade de doenças transmitidas pela água.

Um sistema de abastecimento de água caracteriza-se pela retirada da água da natureza, adequação de sua qualidade, transporte até os aglomerados humanos e fornecimento à população em quantidade compatível com suas necessidades. A portaria nº 518 (BRASIL. Portarias, 2004) define sistema de abastecimento em dois grupos: sistema de abastecimento de água, que se destina à produção e a distribuição canalizada de água potável para populações; e solução alternativa de abastecimento, que é toda modalidade de abastecimento coletivo de água distinto de abastecimento de água, incluindo, poço comunitário e instalações condominiais horizontal e vertical.

As águas subterrâneas oriundas de poços rasos constituem-se importantes fontes de suprimento de água para consumo humano. Na cidade de Maringá, PR., esses poços estão instalados em empresas, hospitais e principalmente em condomínios residenciais. O controle da qualidade desta água está sob responsabilidade do operador do sistema de solução alternativa. Um dos maiores problemas das fontes particulares é a ausência de monitoramento da qualidade da água consumida (MISRA, 1975).

Na cidade de Maringá, a modalidade de tratamento de água empregada pela Companhia Paranaense de Saneamento (Sanepar) no sistema de distribuição é do tipo tratamento completo, que inclui o pré-tratamento da água in-natura que chega a estação (pré-cloração, adição de coagulante e eventual adição de álcali), seguindo para os processos de coagulação, floculação, decantação e filtração descendente. A água segue então para a desinfecção (pós-cloração), fluoretação e correção de pH. Após o tratamento a água é armazenada em 4 reservatórios até sua distribuição.

Água potável de sistemas de distribuição é colonizada por microorganismos heterotróficos (bactérias, fungos, etc.) (LECHEVALLIER et al., 1987; SERVAIS et al., 1992) que crescem a partir da matéria orgânica biodegradável. Microorganismos potencialmente patogênicos (*Legionella* spp, por exemplo) e microorganismos de origem fecal (*Escherichia coli*, por exemplo) podem também encontrar favoráveis condições e proliferar nestes sistemas (FASS et al., 1996; LECHEVALLIER, 1990). A manutenção de desinfetante residual no sistema de distribuição tem o objetivo de produzir condições desfavoráveis para a sobrevivência microbiana na água para consumo. Entretanto, em alguns casos, aplicação imprópria de um desinfetante pode na realidade acentuar as condições de crescimento bacteriano. Ozônio, por exemplo, pode aumentar os níveis de carbono orgânico assimilável e, não estando

acoplado com filtração biológica, pode resultar em aumento do crescimento bacteriano no sistema de distribuição (JANSSENS et al., 1984).

De acordo com LeChevallier et al. (1996) quando 0,2 mg/l de cloro ou valores acima do normal são mantidos, a ocorrência de coliformes é reduzida em cerca de 50%. A portaria de 25 de março de 2004 (BRASIL. Portarias, 2004) preconiza que após a desinfecção, a água deve conter um teor mínimo de cloro residual livre de 0,5 mg/L, sendo obrigatória a manutenção de, no mínimo, 0,2 mg/L em qualquer ponto da rede de distribuição.

Costerton *et al*. (1999), define biofilme como “uma comunidade estruturada de células bacterianas encerradas em uma matriz polimérica produzida por si próprias a uma superfície viva ou inerte”. A presença de biofilmes nas redes de distribuição formados pela presença de matéria orgânica e microorganismos na água é responsável pela demanda de desinfetante. Camper et al. (1986), reporta que organismos ligados a partículas orgânicas são menos susceptíveis a desinfecção, e podem, portanto, passar as barreiras de desinfecção e ter um impacto significativo sobre os biofilmes destes sistemas.

De acordo com as novas diretrizes 98/83/EEC do Conselho da União Européia (1998), água para consumo humano deve ser livre de quaisquer microorganismos e parasitas e de qualquer substância a qual, o número ou a concentração, constitui um perigo potencial para a saúde humana. De acordo com estes dados, a U.S. Enviromental Protection Agency pela primeira vez emprega uma avaliação quanto ao risco microbiológico. Foi definido que um risco anual de 10^{-4} (uma infecção por 10.000 consumidores ao ano) deve ser aceito para doenças adquiridas através de água potável.

Quanto ao padrão microbiológico de potabilidade, fica estabelecido na legislação brasileira (BRASIL. Portarias, 2004) que água para consumo humano em toda e qualquer situação deve ter ausência em 100 ml de *Escherichia coli* ou coliformes termotolerantes, e que em amostras individuais procedentes de poços, fontes, nascentes e outras formas de abastecimento sem distribuição canalizada, tolera-se a presença de coliformes totais em 95% das amostras examinadas no mês, na ausência de *Escherichia coli* e/ou, coliformes termotolerantes, nesta situação devendo ser investigada a origem da ocorrência, tomadas providências imediatas de caráter corretivo e preventivo e realizada nova análise de coliformes.

A preocupação com a qualidade da água de rede pública e, sobretudo, a busca do bem-estar proporcionado pelos sais minerais tem provocado nos últimos anos uma contínua demanda por água mineral, em todos os países. Acompanhando uma tendência mundial, o mercado brasileiro de águas minerais continua em franca ascensão, consolidando-se como um dos setores da economia que mais cresce no país. Somente em 2001, o volume de produção e consumo de águas minerais engarrafadas cresceu 23% em relação ao ano anterior, somando 4,32 bilhões de litros. Com esses resultados, o Brasil já se coloca como o sexto maior mercado mundial de água mineral. No país, o consumo per capita, que era de 24,9 litros em 2001 deverá aproximar-se este ano dos 30 litros/ano, elevando a produção para mais de 5 bilhões de litros. A região Sul ocupa o terceiro lugar no ranking da produção nacional, com 11,3%. O segmento de maior crescimento e consumo continua sendo o de garrafão de 20 litros, que domina 57% do mercado de águas minerais engarrafadas (ABINAM, 2005).

Na Europa, o Conselho da União Européia (80/778/EEC, 1980), estabelece que água mineral natural (incluindo fontes com baixos minerais) não deve conter

nenhum parasita, microorganismos patogênicos, *E. coli* e outros coliformes, estreptococos fecais, bactérias anaeróbicas redutoras de sulfato ou *Pseudomonas aeruginosa* e estipula que 12 horas após o engarrafamento o número total de bactérias não deve exceder 100 UFC (unidade formadora de colônia)/ml. No Brasil, segundo a resolução nº 54 (BRASIL. Resoluções, 2004), a água mineral natural deve estar em conformidade com as seguintes características microbiológicas: ausência de coliformes totais, *Escherichia coli* ou coliformes termotolerantes e enterococos em 100 ml, e também ter ausência de organismos patogênicos como *Pseudomonas aeruginosa* e Clostridios sulfito redutores ou *Clostridium perfringens* em 100 ml. Enquanto padrões microbiológicos existem para águas engarrafadas, o mesmo produto uma vez instalado em um dispensador geralmente não é regulamentado e é raramente controlado (LEVESQUE, 1994).

A quantidade da microbiota de água de fonte é usualmente alta. Águas de nascente contém uma microbiota natural composta principalmente de espécies dos gêneros: *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, *Cytophaga*, *Moraxella* e *Pseudomonas* (TAMAGNINI, 1997). Depois do engarrafamento, o número de contagem de viáveis aumenta rapidamente, alcançando 10^4-10^5 UFC/ml dentro 3-7 dias (LECLERC e COSTA, 1998). Durante as semanas seguintes, a contagem bacteriana diminui lentamente ou permanece constante. No final de 2 anos de estocagem, a contagem colonial ainda fica entre 10^3 UFC/ml (BISCHOFBERGER et al., 1990). Em adição a contaminação natural, o produto pode também ser deteriorado antes de alcançar o consumidor. A contaminação pode ocorrer em qualquer momento durante o processamento (LEVESQUE, 1994).

A transmissão de doenças pelo consumo de água ainda é uma questão de grande interesse, a despeito do esforço mundial e das modernas tecnologias

utilizadas na produção de água potável de boa qualidade. O problema não está confinado aos países em desenvolvimento onde o tratamento pode ser inadequado ou inexistente. Isto também poder ter sérias proporções em países industrializados (KRAMER et al., 1996). Falhas mecânicas, erros humanos, deterioração na qualidade da fonte de água podem levar a falhas mesmo nos melhores sistemas de tratamento e processos de desinfecção (ROEFER et al., 1996). Rápida e confiável rotina de monitoramento da qualidade microbiológica da fonte e da água potável tratada permanecerá de fundamental importância para o controle das doenças transmitidas pela água.

Para proteger o consumidor contra possíveis doenças veiculadas pela água, é importante que a ocorrência e os níveis de microorganismos patogênicos na água para consumo sejam monitorados. Isto pode, entretanto, não ser atingido. Muitos patógenos estão presentes somente em condições específicas e, quando presentes, ocorrem em baixos níveis comparados com outros microorganismos. Muitos métodos empregados para detecção de patógenos não oferecem nenhuma indicação dos níveis de contaminação, mas somente se o patógeno específico está presente ou não (GRAY, 1994).

Como resultado de estudos epidemiológicos, foi concluído que não é sempre necessário isolar ou detectar o organismo patogênico, pode ser suficiente ter um indicador da presença do respectivo patógeno por outros métodos. O método mais importante é a detecção e enumeração de indicadores fecais, que são utilizados para estabelecer a presença potencial de contaminação fecal na água. Essas bactérias indicadoras não são necessariamente patogênicas, mas tem a mesma origem que bactérias patogênicas. Uma possível contaminação da água, indicada pela detecção de *E. coli* ou aumento no número de coliformes, sempre inclui uma

alta probabilidade da ocorrência de patógenos excretados via fezes (SZEWZYK, 2000).

Bactérias do grupo coliforme são consideradas como as pertencentes aos gêneros *Escherichia*, *Citrobacter*, *Enterobacter* e *Klebsiella*, mas o grupo é mais heterogêneo e inclui uma grande variedade de gêneros, como *Serratia* e *Hafnia*. Portanto, o grupo coliforme inclui espécies ambientais e fecais. Apesar de que organismos coliformes não podem ser relatados diretamente à presença de contaminação fecal ou patogênica em água potável, o teste para coliformes é ainda utilizado para monitorar a qualidade microbiológica de sistemas de água tratados. Testes para bactérias do grupo coliforme são padronizados e relativamente fáceis e baratos para usar. Eles são por essa razão mais rapidamente administrados do que testes para determinar a presença individual de microorganismos patógenos em água (THERON e CLOETE, 2002). Coliformes totais devem estar ausentes imediatamente após a desinfecção, e a presença desses organismos indicam tratamento inadequado. A presença de coliformes totais nos sistemas de distribuição e águas engarrafadas pode revelar recrescimento e possibilitar a formação de biofilme ou contaminação através de ingresso de material externo.

Bactérias coliformes que são capazes de fermentar a lactose a 44-45ºC são conhecidas como coliformes termotolerantes. Na maioria das águas, o gênero predominante é *Escherichia*, mas algumas espécies de *Citrobacter*, *Klebsiella* e *Enterobacter* também são termotolerantes. Espécies de coliformes termotolerantes, exceto *E. coli*, pode incluir organismos ambientais. *Escherichia coli* ocorre em altos números em fezes humanas e animais, água de esgoto e água sujeita a poluição fecal recente, por isso é o organismo indicador preferido para este propósito.

Enterococos intestinais são um subgrupo do grupo de organismos definido como estreptococos fecais. Esse grupo foi separado do restante de estreptococos fecais porque são relativos a poluição fecal. A maioria das espécies não se multiplica em ambientes aquáticos e são tipicamente excretados nas fezes de humanos e outros animais de sangue quente. O grupo dos enterococos pode ser usado como um indicador de recente contaminação fecal (WHO, 2004). O uso de enterococos como um indicador de contaminação fecal de água recreacional foi recomendado pela U.S. Environmental Protection Agency (U.S. EPA, 1986). A recomendação foi baseada em estudos que demonstram que enterococos tem uma relação diretamente forte com doenças associadas a banhos em água de mar (LEVIN et al., 1975) ou água doce (DUFOUR, 1984).

Staphylococcus aureus é relativamente difundido no ambiente, mas é encontrado principalmente na pele e membranas mucosas de animais. Faz parte da microbiota normal da pele humana e é encontrado na nasofaringe de 20-30% de adultos em algum momento da vida. *Staphylococcus* são ocasionalmente detectados no trato gastrointestinal e pode ser detectado em água de esgoto. *S. aureus* pode ser liberado por contato humano dentro de ambientes aquáticos como piscinas, e outras águas recreacionais, e também pode ser detectado em sistemas de abastecimento de água (WHO, 2004). Bactérias gram-positivas ocorrem em água mineral natural, porém há evidências convincentes de que são derivadas da fábrica de engarrafamento, por serem habitantes comuns da pele e mucosa dos trabalhadores (LECLERC, 2002), sendo, portanto, um indicador das condições de higiene do processo industrial. Hunter e Burge (1987), encontraram *S. epidermidis* e *S. humanis* em 6 dos 52 galões de água, os quais foram atribuídos a pobres práticas de higiene.

A contagem de bactérias heterotróficas (HPC) detecta um grande espectro de microorganismos heterotróficos. Este espectro detectado inclui organismos sensíveis a processos de desinfecção, como bactérias coliformes; organismos resistentes à desinfecção, como os formadores de esporos e organismos que se proliferam rapidamente em água tratada na ausência de desinfetante residual. Este teste tem pouco valor indicativo para a presença de patógenos, mas pode ser utilizado no monitoramento operacional como um indicador de tratamento e desinfecção. Microorganismos heterotróficos incluem membros da microbiota normal de ambientes aquáticos (tipicamente não patogênicos), e organismos presentes em uma extensão de fontes poluidoras. O número de organismos HPC é reduzido significantemente por práticas de desinfecção, mas em condições apropriadas, como ausência de desinfetantes residuais, estes organismos podem crescer rapidamente. Os principais determinantes do crescimento ou recrescimento em sistemas de distribuição de água ou água mineral depois do engarrafamento são: temperatura, disponibilidade de nutrientes, incluindo assimilação de carbono orgânico, falta de desinfetante residual e estagnação (WHO, 2004).

Água mineral natural não pode ser sujeita a nenhum tipo de desinfecção que modifique ou elimine seus componentes biológicos; por essa razão sempre contém as bactérias que são componentes naturais dessas águas. Ė claro que depois do engarrafamento, a contagem bacteriana deve ser resultante do aumento normal de bactérias presentes na fonte. Quantificação de HPC em água mineral engarrafada é útil, pois prova que não ocorreu desinfecção e ajuda a assegurar que da água da fonte até o produto final não ocorreram maiores mudanças quantitativas microbianas na água (LECLERC, 2002).

HPC pode incluir microorganismos patogênicos ou potencialmente patogênicos, como *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Klebsiella*, *Legionella*, *Moraxella*, *Mycobacterium*, *Serratia*, *Pseudomonas* e *Xanthomonas*. Patógenos oportunistas estão associados com infecções secundárias, com infecções em crianças ou idosos e em pacientes imunocomprometidos. O número de HPC tipicamente obtidos em água potável não deve exceder 100 UFC/ml (regulamentação para água potável Alemã). Em outros países, como África Sul, limites similares foram regulamentados para água potável (HAMBSCH et al., 2004; PAVLOV et al., 2004; SZEWZYK et al., 2000; WHO, 2004). A legislação brasileira (BRASIL. Portarias, 2004), porém, regulamenta que 20% das amostras mensais nos sistemas de distribuição não devem exceder 500 UFC/ml.

Pseudomonas aeruginosa é um patógeno oportunista que é conhecido por causar infecções no trato urinário, infecções sistêmicas respiratórias, infecções gastrointestinais, infecções do sistema nervoso central, infecções de pele, endocardites, otite externa, bacteriemia e uma variedade de infecções sistêmicas, particularmente em pacientes com queimaduras severas e em pacientes imunocomprometidos (ex.: com câncer ou SIDA), infecções crônicas do pulmão em pacientes com fibrose cística, ceratite ulcerativa aguda em usuários de lentes de contato duradouras (LYCZAK, 2000; SHRIVASTAVA, 2004).

Estes organismos crescem bem a 25-37ºC, mas podem crescer lentamente ou ao menos sobreviver em altas ou baixas temperaturas. A habilidade de crescer a 42ºC distingue esta de muitas outras espécies de *Pseudomonas.* Em adição a essa versatilidade nutricional, *P. aeruginosa* resiste a altas concentrações de sais, corantes, anti-sépticos fracos e a maioria dos antibióticos comumente utilizados. Essas propriedades ajudam a entender a ubiqüidade natural desta espécie e

contribui para sua proeminência como causa de infecções hospitalares. Espécies de *Pseudomonas* normalmente habitam solos, água e vegetações e podem ser isoladas da pele, esôfago e fezes de pessoas saudáveis. Freqüentemente colonizam comida hospitalar, pias, torneiras, esfregões e equipamentos respiratórios. A difusão ocorre de paciente para paciente por contato com objetos pessoais ou por ingestão de água ou comida contaminada (SHRIVASTAVA, 2004). A capacidade de produzir diversas e freqüentes infecções é devido a um arsenal de fatores de virulência.

Um aspecto particular de *P. aeruginosa* é a habilidade de crescer em ambientes com poucos nutrientes e utilizar muitos compostos ambientais como fonte de energia. A capacidade de crescer em água mineral com baixos níveis de sólidos dissolvidos e compostos orgânicos confirma esta capacidade. Legnani et al. (1999) isolaram linhagens de *Pseudomonas* na fase final de lavagem de galões em uma fábrica de água mineral e reforça o risco destes microorganismos contaminarem galões, os quais serão abastecidos e colocados a venda. Baixos números de células podem se multiplicar e alcançar rapidamente concentrações nocivas para certas categorias (recém nascidos, idosos, imunodeprimidos, pacientes transplantados). A presença de altos números de *P. aeruginosa* em água potável notadamente, em água engarrafada, pode ser associado com reclamações de gosto, odor e turbidez.

A ocorrência de *P. aeruginosa* em sistemas de distribuição de água indica uma deterioração da qualidade microbiológica da água causada pela alta disponibilidade de nutrientes e baixo fluxo. É um típico organismo formador de biofilmes que cresce em materiais que libere nutriente (SZEWZYK, 2000).

Este microorganismo é sensível à desinfecção e sua entrada dentro dos sistemas de distribuição pode ser minimizada por desinfecção adequada. Medidas tomadas para minimizar o crescimento de biofilmes, incluindo tratamento para

otimizar a remoção de carbono orgânico, restrição do tempo de permanência da água nos sistemas de distribuição e manutenção de desinfetantes residuais, podem reduzir o crescimento destes organismos (WHO, 2004).

Os biofilmes que podem se desenvolver dentro de sistemas de distribuição são compostos de bactérias encerradas em uma matriz polimérica. Podem atuar como uma barreira de difusão lenta a alguns agentes antimicrobianos. Exige uma demanda de cloro, reduzindo a proteção de desinfetantes residuais, além disso, vários agentes antimicrobianos podem ser desativados nas camadas externas do biofilme, antes que alcancem as camadas internas. Os biocidas são menos efetivos contra biofilmes, em relação às células planctônicas (DE BEER, et al., 1994). A importância de *Pseudomonas* é quanto a sua habilidade de viver em biofilmes, onde pode atuar como uma contínua fonte de contaminação, já que difere das células planctônicas em suas características virulentas (SHARMA et al., 2003; LU et al., 1999, SHIRTLIFF et al., 2002).

A produção de slime pode ser um potencial mecanismo bacteriano de resistência contra o cloro (SEYFRIED e FRASER, 1980), Cepas mucóides de *P. aeruginosa* são caracterizadas por uma superprodução de alginato extracelular, o slime que contém alginato confere proteção contra o cloro e pode contribuir para a sobrevivência dessa bactéria em sistemas de água clorados (GROBE et al, 2001).

Bactérias são capazes de crescer em biofilmes em uma grande variedade de superfícies como, tubulações de sistemas de distribuição de água, superfície interna de garrafas de água mineral, cateter urinário e venoso, lentes de contato, entre outras. O primeiro passo para o início da formação do biofilme é a aderência das bactérias planctônicas a uma superfície inerte, e depende das interações entre as células bacterianas e as condições da superfície de interesse (DUNNE Jr., 2002). A

hidrofobicidade da superfície celular dos microorganismos é um importante fator na sua habilidade em aderir superfícies sólidas (JAYASEKARA et al., 1999).

A habilidade de aderir e penetrar em células epiteliais são comuns a maioria dos organismos patógenos, e é considerado o primeiro passo no estabelecimento de infecção no hospedeiro e por isso, um importante fator de virulência. A aderência da bactéria patogênica é um passo crítico na patogênese de muitas infecções, principalmente em casos onde o patógeno está confinado a superfície de mucosas, incluindo o trato respiratório (HIRAKATA et al., 1998).

Pseudomonas aeruginosa é um importante patógeno mais freqüentemente responsável por infecções hospitalares, a prevalência de isolados com múltipla resistência tem aumentado, e é um microorganismo bem conhecido por desenvolver resistência a agentes antimicrobianos durante o curso da terapia. E uma das razões deste microorganismo ser considerado um patógeno perigoso é sua resistência natural a alguns antibióticos. Somente poucos antibióticos são efetivos, incluindo fluorquinolonas, aminoglicosídeos e β-lactâmicos de largo espectro, e estes antibióticos não são efetivos contra todas as cepas (SADER et al., 1993; SHRIVASTAVA et al., 2004, GENCER et al., 2002).

A atividade bactericida de anticorpo e complemento em soro fresco normal humano é considerada como um significante componente da defesa do hospedeiro contra bactérias Gram-negativas. A atividade bactericida do soro pode limitar a invasão ou persistência de organismos na circulação sanguínea. Muitos estudos têm correlatado a resistência ao soro de bacilos Gram-negativos com virulência e patogenicidade (SCHILLER e HATCH, 1983).

Entre os fatores de virulência extracelulares secretados por *P. aeruginosa* estão as hemolisinas, as quais contribuem para a invasão do tecido e danos devido aos seus efeitos citotóxicos.

A detecção de atividade bacteriana em um ou vários dos testes para fatores de virulência deve ser utilizada para demonstrar riscos potenciais à saúde que bactérias isoladas de água potável possuem.

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OBJETIVOS

Avaliar a qualidade bacteriológica de água do sistema de abastecimento municipal, de água de solução alternativa de abastecimento e de água mineral utilizada na casa dos consumidores e de galões novos.

Caracterizar cepas de *Pseudomonas aeruginosa* isoladas dessas diferentes fontes quanto aos seus fatores de virulência:

- Resistência a antibióticos
- Formação de biofilme
- Persistência ao cloro de células planctônicas e biofilme
- Resistência ao soro humano normal
- Citotoxicidade a linhagem de células VERO
- Hidrofobicidade
- Aderência em células epiteliais e materiais abióticos
- Hemólise e hemaglutinação.

Comparison of the bacteriological quality of tap water and bottled mineral water

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Abstract

The bacteriological quality of tap water from municipal water supplies, 20-L bottles of mineral water from water dispensers and samples collected from new 20-L bottles mineral water were comparatively studied. Heterotrophic plate count, total coliforms, termotolerant coliforms, *Escherichia coli*, fecal streptococci, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* were enumerated. The results showed that 76.6% and 68.1% of the 20-L bottles from water dispensers and unoponed bottles of mineral water, respectively, were contaminated by at least one coliform or indicator bacterium and/or at least one pathogenic bacterium. The proportion of tap water samples from municipal water system contaminated was 36.4%. The bacteriological quality of municipal tap water is superior when compared to the 20-L bottles mineral water collected from water dispensers and samples collected from new 20-L bottles mineral water before installation in the dispensers. This highlights the need for an improved surveillance system for the bottled water industry. For the municipal water systems, it is recommended to perform the *Pseudomonas* enumeration periodically, in addition to the routine data collected by most system.

***Key words*:** Bottled mineral water. Tap water. Coliform. Streptococci. *Staphylococcus aureus. Pseudomonas aeruginosa*

1 Introduction

The transmission of waterborne diseases is still a matter of major concern, despite worldwide efforts and modern technology utilized for the production of safe drinking water (Venter, 2000). This problem is not confined to the developing world where water treatment may not exist or are inadequate. There may also be contamination during storage, a lack of regulations and limited understanding and awareness among the population (AAM, 1996). It may also assume serious proportions in industrial countries (Kramer et al., 1996). Mechanical failure, human error or deterioration of the quality of the source water can lead to the failure even in the best treatment systems and disinfection processes (MacKenzie et al., 1994; Roefer et al., 1996).

The water quality is often related to the degree of bacterial contamination. Drinking water distribution systems are colonized by saprophytic heterotrophic microorganisms that grow on biodegradable organic matter (Servais et al., 1992). Potentially pathogenic microorganism (e.g., *Pseudomonas aeruginosa*) and microorganisms of fecal origin (e.g., *Escherichia coli*) may also find favorable condition and proliferate in these systems. The quantity of bacteria in commercial mineral water is generally dependent on the disinfecting process of natural spring water use at the factory. It is well known that natural mineral water is characterized by its bacterial flora, chemical and physical composition. The quantity of microbial flora of spring water is usually high. Spring water contains a natural microbiota composed mainly of species of the genera *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, *Cytophaga*, *Moraxella*, and *Pseudomonas.* If these microorganisms are not adequately removed during processing and bottling, bacterial

multiplication may occur for 1 to 3 weeks after bottling, and the bacterial count can reach 10^3 to 10^4 bacteria per ml at 37°C (Tamagnini and Gonzales, 1997). In addition to natural contamination, the product can also be deteriorate before it reaches the consumer.

The purpose of the present study was to compare the bacteriological quality of tap water from municipal water supplies, 20-L bottles of mineral water from water dispensers and samples collected from new 20-L bottles mineral water before installation of the bottles in the dispensers.

2 Materials and methods

2.1. Water samples

Residences and workplaces were randomly selected from the list of the Companhia de Saneamento do Paraná of the Maringá city, Paraná State, Brazil. To be included in the study, owners of water dispensers had to have 20-L bottle mineral water supplied by a recognized company. Then, 20 residences and 20 workplaces served by municipal water system were selected. In addition, water samples were collected for bacterial analysis from unopened 20-L bottles of mineral water representing the nine bottling companies sampled in the residences and workplaces. The samples for bacteriological analysis were collected in sterilized plastic bottles with sodium thiosulfate (10%) and transported to the laboratory in ice. Analyses were carried out within 4 h of sampling.

2.2 Culture media

The microbiological parameters determined were aerobic and facultative anaerobic heterotrophic bacteria (HPC), total (TC) and fecal coliforms (FC), fecal

streptococci (FS), *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The heterotrophic plate count (HPC) was determined by the pour plate technique as described by the Standard Methods (APHA 1995). TC, FC, FS, *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* were quantified by membrane filtration. Media for bacterial analyses were obtained from Difco Laboratories, Detroit, MI, or from Becton Dickinson and Company, Cockeysville, MD. The solid media employed, the bacteria enumerated and conditions for bacterial enumeration were as follows: m-Endo- Les, total coliforms at 35ºC for 24 h; m-FC, fecal coliforms at 44,5ºC for 24 h; m-TEC, *E. coli* at 35ºC for 2 h and 44,5ºC for 22 h; m-E, fecal streptococci at 41ºC for 48 h*;* m-PAC, *P. aeruginosa* at 41,5ºC for 24 h; and Baird Parker, *S. aureus* at 35ºC for 48 h, Plate Count Agar, HPC at 35º for 72 h. Confirmation consisted of EIA at 41ºC for 20 m and Milk at 35ºC for 24/48 h, cytochrome oxidase test, Gram stain and BBL Crystal Identification Systems (Becton Dickinson).

2.3 Chemical analyses

The physico-chemical quality of the water was measured in terms of pH, chlorine levels, total nitrogen, total carbon, total organic carbon, inorganic carbon, and phosphorus (APHA , 1995).

2.4 Statistical analysis

Results were analyzed by linear regression and *t*-test, at $p < 0.005$ of confidence level.

3 Results and discussion

The mean values of total nitrogen, total carbon, total organic, carbon inorganic carbon, phosphorus, pH, and chlorine are displayed in Table 1. The standards for microbial quality of water for human consumption in Brazil are shown in Table 2. The results of the microbiological analysis performed on samples of tap water from municipal water supplies, 20-L bottles of mineral water from water dispensers and samples collected from new 20-L bottles mineral water before installation of the bottles in the dispensers appear in Table 3. The results of heterotrophic plate count (HPC) enumerated by pour plate technique for the mineral water from dispensers, tap water, and the unopened bottle samples are shown in Table 4. Table 5 shows comparison among the absence of one indicator with the absence of all the other indicators.

The physico-chemical characteristics of the bottled water and tap water were compared with the US EPA (2001) drinking water standards. The US EPA standards for drinking water were used since some guideline levels for physic-chemical characteristics were lacking in the Brazilian standards. All water quality constituents in bottled mineral water and tap water analyzed were within acceptable limits when compared with the US EPA directives. In most case, the 20-L bottles of mineral waters had higher values of total carbon, inorganic carbon and phosphorus than the tap waters. There were similarities in the concentration of total nitrogen and total organic carbon, as well as in pH determined for the two types of water. The mean value of chorine for the tap water from municipal water supplies is 3 times the minimum concentration used to disinfect drinking water supplies in Brazil.

The Brazilian directives (ANVISA, 2004) regulate water from municipal water supplies on the basis of coliform content and heterotrophic plate count, whereas

more stringent bottled mineral water regulations prohibit the presence of a group of potentially pathogenic bacteria (*Pseudomonas*, fecal Streptococci and Clostridia).

Total coliform bacteria were detected in 31 out of 77 (40.2%), 5 out of 22 (22.7%), and 3 out of 96 (3.1%) of the bottled mineral water, new bottles mineral water, and tap water samples from municipal supplies, respectively. Eight (10.3%) of the bottled mineral water, three (3.1%) of the tap water and one (4.5%) of new bottles mineral water samples were positive for fecal coliforms. Five (6.4%) samples contaminated with *Escherichia coli* were bottled mineral water from water dispensers.

Coliform organisms have long been recognized as a suitable microbial indicator of drinking-water quality, largely because they are easy to detect and enumerate in water (WHO, 1993). In drinking water from municipal supplies, the coliform test can be used as an indicator of treatment efficiency and of the integrity of the distribution system. Although coliform organisms may not always be directly related to the presence of fecal contamination, the presence of coliforms in drinking water suggests the potential presence of pathogenic enteric microorganisms such as *Salmonella* spp, *Shigella* spp, and *Vibrio cholerae*. Coliform bacteria are the only microbiological contamination to be regulated by law in both tap and bottled water. For tap water, the Brazilian directives states that a public water supplies that test at least 40 samples per month must be not present in 95% is samples taken throughout any 12-month period. In the case of minimum frequencies, one sample every week for waterworks with surface water source and one sample every 2 weeks for waterworks with a ground water source must not be detectable in any 100-ml sample.

The finding that 31/77 or more than 1/3 of the bottled water contain coliform organisms suggests the need for an improved surveillance system for the bottled

water industry. Similar results were observed in new bottle mineral water, 5/22 or almost 1/4 bottles of water were positive for coliforms.

The presence of *E. coli* in water is nearly always associated with recent fecal pollution and it is the preferred indicator organism for this purpose (APHA, 1995). In the present study, the occurrence of coliform bacteria was significantly higher than the isolation of *E. coli*. However, the isolation of *E. coli* from 6.4% of the mineral water samples, with the positive samples being all mineral water from water dispensers, also has health implications, as these microorganisms are considered as indicator of fecal pollutions. Brazilian directive recognize *E.coli* as the best indicator of fecal contamination, since several culture media, which enumerate fecal coliforms may also enumerate *Klebsiella* spp*.,* which are often present in industrial wastes.

Fecal streptococci were detected on the EIA plates in 7 out 77 (9.0%) and 1 out of 22 (4.5%) of the bottled and new bottles of mineral water, respectively. According to WHO (1993), the term “fecal streptococci” refers to those streptococci generally present in the feces of humans and animals. Their primary value in water quality examination is therefore as additional indicators of treatment efficiency.

The fact that neither *E.coli* nor fecal streptococci was found in any of the 96 tap water samples in this study suggests the absence of fecal contamination of these samples. However, the finding that 3.1% of the tap water sampled in the current study failed to meet the Brazilian standard for both fecal and total coliforms in drinking water should therefore be of concern.

Other bacteria isolated from water samples included *Staphylococcus aureus,* which was recovered from 25 out 77 (32.4%), 2 out 22 (9.0%), and 6 out 96 (6.2%) of the bottled mineral water, new bottles and tap water samples, respectively.

Pseudomonas aeruginosa contamination was evident in 43% of the samples; over two third (58.4%) of the bottled mineral samples were contaminated, which was higher than the 20-L new bottles mineral water (50%) and tap water samples from municipal supplies (29.1%).

P. aeruginosa is an opportunistic pathogen that is known to cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, and in cancer and AIDS patients who are immunocompromised. Outbreaks caused by this organism have been reported in various settings. The strain responsible for the outbreak may be spread via the hands of healthcare workers or by environmental sources of transmission such as contaminated water (Kolmos et al., 1993; Richard et al., 1994).

A particular feature of *Pseudomonas aeruginosa* is its ability to grow in low-nutrient water. Beside being a primary cause of disease, *P. aeruginosa* is often monitored as an indicator of other bacterial contamination of fecal origin (Warburton, 1992). The public health significance of the high number of *Pseudomonas* spp, which develop is unclear. Many *Pseudomonas* spp recovered from water are resistant to antimicrobial agents (Hernandez Duquino and Rosenberg, 1987).

The number of samples with heterotrophic plate count over the maximum level legally permitted in Brazil (500 colony forming units ml^{-1}) were 4 (4.17%), 67 (87.01%) and 10 (45.46%) for tap water, mineral water and new bottles of mineral water, respectively. Heterotrophic plate count ranged from 14 to 300000 CFU ml^{-1} among the 77 bottled water examined, including 67 samples with levels above 500 CFU ml^{-1}. In addition, bacterial count in samples of 20-L unopened bottles of mineral water ranged from 2 to 226000 CFU ml^{-1}, counting 10 (45.46%) samples with levels

above 500 CFU ml^{-1}. Of the 96 tap water samples from municipal supplies, only 4 had bacterial count above 500 CFU ml^{-1}. Considering that heterotrophic plante count are indicator of hygienic condition and that the disinfection do no eliminate completely these bacteria, different ranges of total bacterial densities were established.

According to the Brazilian regulations, disinfection or sterilization of commercially available mineral water is not permitted. Therefore, they generally have high heterotrophic plate counts (HPC) a few days after bottling that should result only from an increase of bacteria present in the source water. The number of bacteria recovered at the source is generally very low, around 10 CFU ml^{-1}, but there are many report that viable counts increase, notably in uncarbonated water, to 10^4-10^5 CFU ml^{-1} after 1-2 weeks of storage (Tamagnini and Gonzales, 1997; Bischofberger et al., 1990; Mavridou, 1992; Mavridou et al., 1994; Tsai and Yu, 1997).

On the basis of results obtained with a sampling of initial streams of water, the bacteriological quality of municipal tap water is superior to the quality of 20-L bottles of mineral water from water dispensers and samples collected from new 20-L bottles mineral water before installation of the bottles in the dispensers. Of the 195 samples examined, 109 (55.8%) were contaminated by at least one coliform or indicator bacterium and/or at least one pathogenic bacterium, including 59 (76.6%) of the 77 bottled mineral water from dispenser, 35 (36.4%) of the 96 tap water from municipal supplies, and 15 (68.1%) of the 22 unopened bottles of mineral water. No *Clostridium* was found in any of the 195 samples.

When the bacteria associated with fecal contamination and those derived from environmental source were considered, the results shows that the absence of *P.*

aeruginosa produced the highest percentage of negative samples for the other indicators in tap water (89.7%) and bottled mineral water (56.2%).

In addition, a correlation matrix was established to compare the degree of association among microbiological indicators used in this study (data not shown). A positive correlation was observed among *P. aeruginosa*, fecal coliform and *S. aureus*, as well as between total coliform and *E. coli* for samples of mineral water. Contrarily, no correlation between the bacteria associated with fecal contamination and those derived from environmental source was observed for samples of tap water from municipal water supplies.

This highlights the need for an improved surveillance system for the bottled water industry. Obviously, better efforts are necessary to eliminate planktonic bacteria and the biofilm, both sources of contamination that can concentrate opportunistic pathogens like *P. aeruginosa.* In addition, frequent cleaning of water dispensers would help eliminate various contaminants from the water and therefore lower the possibility of waterborne illness.

For the municipal water systems, the enumeration for *Pseudomonas* should be performed periodically (e.g. once a week or once a month depending on the system size and analytical capabilities), in addition to the routine data collected by most system (coliforms, total bacteria, chlorine, pH, etc.).

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Table 1

Physico-chemical characteristics of the bottled mineral water and tap water from municipal supplies

Parameter	Mean values (range)	
	Mineral water[a]	Tap water[b]
Total nitrogen (mg l^{-1})	0.65 (0.06-1.29)	0.64 (0.51-0.76)
Total carbon (mg l^{-1})	10.54 (7.68-15.53)	6.80 (6.43-7.16)
Total organic carbon (mg l^{-1})	0.91 (0.76-1.13)	1.01 (0.85-1.16)
Inorganic carbon (mg l^{-1})	9.63 (6.91-14.7)	5.79 (5.58-6.00)
Phosphorus (μg l^{-1})	64.88 (9.40-123.85)	12.20 (10.87-13.53)
PH	7.4 (5-8.5)	7.4 (7-8.1)
Chlorine (mg $l^{-1)}$		0.64 (0-1.5)

[a]20-liter bottles; [b] The most-often-used faucet

Table 2

Brazilian microbiological limits[a] for mineral water and tap water from municipal supplies

Characteristic	Mineral water	Tap water
Heterotrophic plate count[b]	500	500
Total coliforms	0	0
Fecal coliforms	0	0
Escherichia coli	0	0
Staphylococcus aureus	-	-
Pseudomonas aeruginosa	0	-
Fecal streptococci	0	-
Sulfite-reducing clostridia	0	-

[a]Colony-forming units per 100 ml
[b]Colony-forming units per ml

Table 3

Microbiological quality of tap water and bottled mineral water

Indicator bacteria or pathogen	Number (%) of samples positive		
	Tap water[a] n=96	Mineral water[b] n=77	New bottles[c] n=22
Total coliforms	3 (3.1)	31 (40.2)	5 (22.7)
Fecal coliforms	3 (3.1)	8 (10.3)	1 (4.5)
Escherichia coli	0 (0.0)	5 (6.4)	0 (0.0)
Fecal streptococci	0 (0.0)	7 (9.0)	1 (4.5)
Pseudomonas aeruginosa	28 (29.1)	45 (58.4)	11 (50.0)
Staphylococcus aureus	6 (6.2)	25 (32.4)	2 (9.0)
Unacceptable microbiology	35 (36.4)	59 (76.6)	15 (68.1)

[a] The most-often-used faucet
[b] 20-liter bottles
[c] Bottled mineral water before installation of the bottles on water dispensers.

Table 4

Range of the heterotrophic plate count (HPC) of tap water and bottled mineral water

HPC (no. of bacteria ml^{-1})	Number and percentage		
	Tap water[a]	Mineral water[b]	New bottles[c]
<1	47 (49.0)	0 (0.0)	1 (4.5)
1-500	45 (46.9)	10 (13.0)	11 (50.0)
>500	4 (4.2)	67 (87.0)	10 (45.5)
Range	0-2350	14-300000	2-226000
Median	95	22658	12665

[a] The most-often-used faucet
[b] 20-liter bottles
[c] Bottled mineral water before installation of the bottles on water dispensers.

Table 5

Comparison among the absence of one indicator with the absence of all the other indicator.

Indicators bacteria	% of negative samples for the other indicators	
	Tap water[a] n=96	Mineral water[b] n=99
Total coliforms	65.6	39.1
Fecal coliforms	65.5	26.1
Escherichia coli	63.5	25.0
Fecal streptococci	63.5	25.7
Pseudomonas aeruginosa	89.7	56.2
Staphylococcus aureus	67.7	34.6

[a] The most-often-used faucet
[b] 20-liter bottles

Characterization of potential virulence markers in *Pseudomonas aeruginosa* isolated from drinking water

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ABSTRACT

The aim of this study was to determine the possible differences among *Pseudomonas aeruginosa* isolates from tap water, mineral water, and artesian well water in relation to their ability to produce different potential virulence factors or markers such as hemolysins, hemaglutinins, and citotoxins, and their ability to adhere to epithelial cells and to abiotic surfaces. The susceptibility to antibiotics, human serum sensitivity and the survival of *P. aeruginosa* isolates in a chlorinated environment were also examined. Of the 30 isolates tested, 16 possessed the capacity to adhere to abiotic surface, 28 to adhere to epithelial cells, while 30 were capable of producing hemolysins, 27 produced cytotoxin, 9 hemagglutinins, and 18 were classified as serum-resistant. For the lowest concentration of chlorine (0.2 mg/l) tested, no killing of biofilm bacteria could be discerned even after prolonged exposure to the agent. Although all drinking water isolates were susceptible to aztreonam, cefepime, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin-tazobactam, and polymyxin, the *P. aeruginosa* isolates have demonstrated resistance to one or more antibiotics. The increasing prevalence of resistance in the isolates from environmental sources may have important therapeutic implications. A notable fraction of *P. aeruginosa* isolates from drinking water were able to elaborate virulence factors, and that the incidence of virulence properties was not statistically different among the three sources. A more extensive study about the virulence properties of this bacterium by toxic assays on animal should be explored. Still more interesting would be toxicity assays on immunodeficient animals and collect information on people drinking waters containing *P. aeruginosa*.

Keywords**:** *Pseudomonas aeruginosa*, drinking water, virulence factors.

1 INTRODUCTION

Pseudomonas aeruginosa is a clinically significant opportunistic pathogen that is known to causes urinary tract infections, respiratory system infections, soft tissue infections, dermatitis, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, and in cancer and AIDS patients who are immunocompromised. Outbreaks caused by this organism have been reported in various settings. The strain responsible for the outbreak may be spread via the hands of healthcare workers or by environmental sources of transmission such as contaminated water (Kolmos et al., 1993; Richard et al., 1994). A particular feature of *P. aeruginosa* is its ability to grow in low-nutrient water. Besides being a primary cause of disease, *P. aeruginosa* is often monitored as an indicator of other bacterial contamination of fecal origin (Warburton, 1992; Clesceri et al., 1998). The public health significance of the high number of *Pseudomonas* spp which develop in water is unclear (Hunter, 1993). Many *Pseudomonas* spp recovered from water are resistant to antimicrobial agents (Hernandez-Duquino and Rosenberg, 1987).

Pseudomonas aeruginosa usually posses virulence-associated factors, such as the ability to produce cytotoxins, hemolysins, proteases, and invasive ability. There is experimental and epidemiological evidence for the existence of multiple mechanisms leading to colonization, and induction of cytotoxicity, pathology and mortality during *P. aeruginosa* infections in vitro (Sawa et al., 1998, Bertrand et al., 2001, Roy-Burman et al., 2001).

The aim of this study was to determine the possible differences among *P. aeruginosa* isolates from tap water, mineral water, and artesian well water in relation to their ability to produce different potential virulence factors or markers such as

hemolysins, hemaglutinins, and citotoxins, and their ability to adhere to epithelial cells and to abiotic surfaces. The susceptibility to antibiotics, human serum sensitivity, and the survival of *P. aeruginosa* isolates in a chlorinated environment were also examined.

2 MATERIALS AND METHODS

2.1 *Bacterial isolates*

A total of 30 *P. aeruginosa* isolates were obtained from tap water, bottled mineral water, and artesian well water of the Maringá city, Paraná, Brazil. Of these, 15 isolates were obtained from unchlorinated drinking water (mineral water and artesian water) supplies. To comparison, type strains ATCC 15442 (*P. aeruginosa* isolated from animal room water bottle), ATCC 27853 (*P. aeruginosa* isolated from blood), and *P. aeruginosa* CI, C II, and C III isolated from blood, sputum and wounds, respectively, were included in all assays. The drinking water isolates were recovered by standard procedures for screening for *P. aeruginosa* in drinking water (APHA, 1995). Typical colonies on m-PAC agar (BBL Microbiology Systems, Cockeysville, Md) and Milk agar (Difco Laboratories, Detroit, Mich) were confirmed for oxidase production. Presumptive *P. aeruginosa* isolates were confirmed with the BBL Crystal Identification Systems (Becton Dickinson).

2.2 *Hemolysis and Hemagglutination assay*

Hemolytic assays was determined by measuring the zone of hemolysis around colonies on blood agar plate as described in earlier report (Imzilm et al. 1996), except

that instead of human blood, 5% sheep erythrocytes in brain heart infusion (BHI) agar (Difco, Detroit, Mich) was used in this study.

The hemagglutination was performed by modification on the method of Burke et al., (1984). Isolates were streaked on Trypticase soy agar plates (Difco) and incubated at 37°C for 16 to 18 h. Two loopfuls of bacteria were emulsified in 0.5 ml of sterile 50 mM sodium phosphate buffer (pH 7.4) containing 0.15M NaCl (PBS), which provided an approximative concentration of 10^{10} to 10^{9} organisms per ml. An equal volume (50 μl) of each sample was mixed with a 4% suspension of washed sheep erythrocytes placed on a microscope slide. A negative control consisting of erythrocyte suspension with PBS was included in each assay. Hemagglutination was recorded positive if the reaction occurred with 30 min.

2.3 *Cytotoxicity assay*

The cytotoxicity assay was carried out, with some modifications, as previously described (Skehan et al., 1990; Skehan, 1995). Briefly, each *P. aeruginosa* isolate was inoculated into 5 ml of Trypticase soy broth, and the mixture was incubated for 16 to 18 h at 37°C. Following centrifugation at 10,000 x g for 30 min at 4°C. Supernatant fluid was carefully removed, filtrated and a ten-fold serial dilution of the supernatant was delivered to each well of confluent Vero cell monolayers grown in 96-well cell culture plates, which was then incubated for 48 h at 37°C under a 5% CO_2 atmosphere. At that time, cultures fixed with 10% trichloroacetic acid for 1 h at 4°C were stained for 30 m with 0.4% sulforhodasmine B (SRB) in 1% acetic acid, and subsequently washed 5 times with deionised water. Bound SRB was solubilised with a 150 μl 10 mM unbuffered Tris-base solution. Absorbance (530 nm) was read in a 96-well plate reader. The dye was removed by four washes with 1% acetic acid.

Protein-bound was extracted with 10 mM Tris. The cytotoxicity was expressed as a percentage of the optical density of the control.

2.4 *Adherence assays*

2.4.1 *Epithelial cells adherence assay*

Human buccal epithelial cells (BEC). Buccal epithelial cells were collected from healthy human volunteers by gently rubbing the inside of the cheeks with sterile swabs which were then agitated in PBS. Epithelial cells were washed twice in PBS to remove unattached microorganisms and were resuspended in PBS at a concentration of 10^5 cells/ml. Standardized suspension of epithelial cells (250 μl) and bacteria (250 μl of an inoculum of $1x10^8$ CFU/ml) were mixed in small screw-capped bottles and incubated for 1 h at 37°C, under agitation at 100 rpm. The mixture was then centrifuged for 10 min at 1000 rpm and washed with PBS to remove unattached bacteria. The final clot was suspended in 50 μl of PBS at pH 7.2. The suspension was spread out on slides and air-dried, fixed with absolute methanol and stained by the Gram procedure. The adherence was expressed as the mean of value of the adherent b bacteria by counting 100 living epithelial cells. (Boussard et al., 1989).

Vero cells. Vero cells were grown in Dulbecco's Minimal Essential Medium (D-MEM), to confluent monolayers on glass coverslips in the bottom of 24-well tissue culture plates (Falcon). Approximately $1x10^7$ bacteria from an overnight culture diluted in D-MEM were added to each well containing a monolayer (≈ 30 bacteria per epithelial cell), and the plates were incubated for 1 h at 37°C, with constant shaking. Coverslips were carefully removed and washed in PBS, pH 7.2, with light manual shaking. The coverslips with the adhered cells were fixed in absolute methanol for 30 min and air-dried. The cells were stained by Gram method and mounted on slide with

synthetic resin (Araldite 502). Each experiment was carried out in triplicate. Areas with discontinuous monolayers were not considered for counts. Five fields for 100 cells per slide were counted. For comparative purposes in some experiment, the coverslips with the adhered cells were processed for scanning electron microscopy as indicated by Tanaka (1989) with the following modifications. After fixation, small drops of the sample were placed on a specimen support with poly-L-lysine. Postfixation was carried out with 1% osmium tetroxide in cacodylate buffer containing 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ for 30 min, with 1% tannic acid in cacodylate buffer for 30 min and with 1% osmium tetroxide for 30 min. Subsequently, the samples were dehydrated in graded ethanol, critical-pointdried in CO_2, coated with chromium in a Penning sputter system in a high-vacuum chamber (Gatan-Model 681), and observed in a JEOL-JSM-6340F field emission scanning electron microscope. Images were obtained using secondary electrons.

2.4.2 *Adherence to abiotic surfaces*

Adherence to abiotic surface was studied with three different materials, glass, silicone and polyethylene terephtalate (PET) in the bottom of 24-well tissue culture plates (Falcon). To prepare the silicone surface, glass coverslips were coated with silicone and incubated overnight to 4°C. One milliliters of an overnight culture of each isolate were added to wells and incubated for 1 h at 37°C with light manual shaking. Coverslips were carefully removed and washed in PBS, pH 7.2, with light manual shaking. The coverslips with the adhered cells were fixed in absolute methanol and air-dried. The cells were stained by Gram method and mounted on slide with synthetic resin (Araldite 502). The adherence was expressed as the mean of value of the adherent bacteria by counting 100 fields.

2.5 *Hydrophobicity assay*

For the hydrophobicity assay, each *P. aeruginosa* was inoculated into 5 ml of Trypticase soy broth for 16 to 18 h at 37°C. The bacteria were then harvested by centrifugation, washed twice and suspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 0.15M NaCl to an absorbency of 0.4 at 660 nm. The bacterial suspension was treated with xylene (2.5:1 v/v; Merck). Following 20 min preincubation at room temperature, the mixture was vigorously mixed for 2 min. After allowing 20 min for hydrocarbon phase to rise completely, the aqueous phase was carefully removed with a Pasteur pipette. The absorbency was then determined at 660 nm against a blank consisting of sodium phosphate buffer. The hydrophobicity indexes (CSH) were calculated as previously described (Rosenberg et al., 1980; Nagao and Benchetrit, 1999).

2.6 *Biofilm formation*

Overnight cultures were inoculated 1:100 into fresh medium. In the microtiter plate assay, inoculated cultures were grown in a 96-well polystyrene microtiter plate (Oliveira-Garcia et al., 2003). Growth of planktonic cells was determined by absorbance at 600 nm. Biofilm was measured by discarding the medium, rinsing the wells with water (three times), and staining bound cells with crystal violet (BBL). The dye was solubilized with 95% ethanol (Merck), and absorbance at 530 nm was determined using a microtiter plate reader. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated controls. All comparative analyses were conducted by incubating strains within the same microtiter plate to minimize variability.

2.7 *Growth and biofilm development*

When performing viable plate counts, growth of planktonic cells was removed, diluted accordingly and plated on nutrient agar (Pitts et al., 2003). After adding 200 μl of PBS to the same wells, the wells were scraped down with a sterile wooden applicator stick for 30 s to dislodge attached cells. The buffer-cell suspension was aspirated and added to 4.8 ml of PBS containing 0.1% Tween (Inlab, São Paulo, Brazil). The wells were washed down once more with 200 μl of these dilute suspensions, then scraped until dry with a sterile applicator stick. The stick was stirred vigorously in the 5 ml buffer/cell volume to dislodge biofilm. Controls and test solution were diluted accordingly and plated on nutrient agar.

2.8 *Chlorine sensitivity tests*

Bactericidal effect. The bactericidal effect of chlorine on *P. aeruginosa* isolates was determined using the method of Seyfried and Fraser (1980) with the following modification. Each *P. aeruginosa* isolated was inoculated into 5 ml of Trypticase soy broth for 16 to 18 h at 37°C. The organisms were centrifuged and washed twice in sterile chlorine demand-free buffered (pH 7.4) water. The washed bacteria were resuspended in the buffer and adjusted to give a standard bacterial suspension of approximately $1x10^7$ CFU/ml. The test system, held in 500 ml screw-cap flasks, contained 150 ml of buffered chlorine demand-free water. Appropriate quantities of chlorine solution were added to each flask and, after thorough mixing, a 50 ml aliquot was removed for free chlorine determination. The test flasks were incubated with 10 ml of standard bacterial suspension and, after a 1 min contact period, 5 ml of sterile sodium thiosulfate solution (0.7 mg/ml) was added to neutralize the chlorine and

thereby stop the reaction. Control and test solution were diluted accordingly and plated on nutrient agar. Plates were counted after 48 h of incubation at 37°C.

Killing of Pseudomonas aeruginosa isolates by chlorine. Planktonic cells and biofim formed were obtained as described above. For dose-dependent determination of killing, planktonic cells was dispensed into 96-well microtiter plates an incubated with appropriate quantities of chlorine solution. After a 5, 10, 15 and 30 min contact period, sterile sodium thiosulfate was added to neutralize the chlorine and thereby stop the reaction. Control and test solution were diluted accordingly and plated on nutrient agar. Plates were counted after 48 h of incubation at 37°C. Similar treatment was performed with biofilm formed in the same wells. After appropriate contact period, all wells were rinsed four times with buffered chlorine demand-free water immediately prior to any analysis. After adding 200 μl of PBS to the wells, the wells were scraped down with a sterile wooden applicator stick as described above. Controls and test solution were serially diluted and plated on nutrient agar.

2.9 *Antibiotic susceptibility*

The antibiotic susceptibility test was performed by the standard disc diffusion method (NCCLS, 2003, 2004). The following antibiotics were used: Trimethoprim-Sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), cefotaxime (30 μg),cefepime (30 μg),ceftazidime (30 μg), piperacillin – tazobactam (100/10 μg), and polymyxin B (300 μg). Organisms were reported as either resistant, intermediate or sensitive to each anti-microbial tested.

2.10 *Serum sensitivity*

Blood samples were drawn from volunteers. Upon collection, blood samples were allowed to clot and separated. Serum aliquots were stored at −70 °C until used for the assay. For these experiments, each *P. aeruginosa* was inoculated into 5 ml of Trypticase soy broth for 16 to 18 h at 37°C. The organisms were centrifuged and washed twice in PBS. The washed bacteria were resuspended in the buffer and adjusted to give a standard bacterial suspension of approximately $1x10^4$ CFU/ml. The assay was carried out in 96-well microtiter plates essentially by method of DeMatteo et al. (1981). To each well added 100 μl of the bacterial suspension and 100 μl of pooled normal fresh human serum at final concentration ranging from 0.1 to 50%. The mixtures were then incubated at 37°C for 1 h with gentle agitation. After the incubation, 10 μl and 100 μl from each well were plated onto BHI agar, incubated overnight at 37°C and the number of CFU/ml determined. The percent of bacterial survival was determined by comparing the number of CFU/ml in each test well, with the number of CFU/ml in the control well (PBS + bacteria). The lower concentration of human serum, which caused >90% reduction in bacterial survival was recorded.

2.11 *Statistical analysis*

Results were analyzed by linear regression and *t*-test, at 5% of confidence level.

3 RESULTS AND DISCUSSION

3.1 *Hemolysis assays and Hemagglutination assays*

The results show that all environmental isolates, clinical strains and type strains ATCC included in this study displayed hemolytic activity against sheep erythrocytes (Table 1). Hemagglutination was observed in 6 of 10 and 3 of 10 isolates from mineral water and tap water, respectively, while only one of the type strains ATCC included in this study showed ability to hemagglutinate sheep erythrocytes. This is particularly noteworthy because this strain was isolated from animal room water bottle.

P. aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion and dissemination (Pavlovskis and Wretlind, 1979; Nicas and Iglewski, 1985; Rumbaugh et al., 1999). Two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa*, may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects (Ostroff and Vasil, 1987).

3.2 Cytotoxicity assay

Majority of the environmental isolates showing cytotoxicity for the cell line tested. Only 2 of the 10 strains of artesian water did not shown cytotoxicity activity. Majority of the isolates showed cytotoxic activities in culture filtrate dilutions at titer of 1/100 or lower (Table 2).

3.3 *Adherence to human buccal epithelial cells and Vero cells*

The ability of *P. aeruginosa* isolates to adhere epithelial cells was investigated using human buccal epithelial cells and Vero cells (Table 3). In both cells, a difference in the abilities of the individual isolates to adhere was observed. However, no general relation between the origin of isolation and the ability to interact with epithelial cells could be established. Thus, the overall ability of isolates of environmental origin to adhere epithelial cells was observed to be no less than of the group of collections strains (clinical and type strains). Three patterns of adhesion, diffuse (Fig. 1B), localized (Fig. 1C) and aggregative (Fig. 1D) were observed. In the diffuse pattern, adherent bacteria were randomly and individually dispersed at the Vero cell surface. The localized and aggregative patterns were characterized by the formation of adherent microcolonies corresponding to small or huge clusters of bacteria, respectively. The tested bacteria (including the group of collections strains) revealed predominantly localized-type adherence (date not shown).

Further visual understanding of cellular factor associated with the adherence of *P. aeruginosa* to Vero cell was gained through scanning electron microscopy (Fig. 1E-F). Subconfluent Vero cell images (Fig. 1E) show the basal extension of connections with the bacteria. Confluent Vero cell images (Fig. 1F) show the general lack of extensions to the bacteria, with the exception of few connections between cells.

Adherence to epithelial cells is an important initial event in the colonization of mucosal surfaces by microorganisms. Strain-to-strain difference with respect to interactions with epithelial cells has been reported in previous studies (Bartkova and Ciznar, 1992; Hostacka and Majtan, 1992; Rajan et al., 2000). However, when

comparing the group of environmental and clinical strains, our results reveal that, with respect to adhesion of epithelial cells, *P. aeruginosa* isolates from drinking water in general do not differ from clinical strains.

3.4 *Adherence to abiotic surface*

Bacterial colonization of solid surface has been demonstrated in a wide variety of environments. The importance of avoiding bacterial adhesion to surgical implants (Costerton et al., 1987; Leake et al., 1982), tap water distribution (Herson et al., 1987), and cooling (Bott and Miller, 1983) is generally recognized.

Table 4 shows the adhesion of *P. aeruginosa* isolates to abiotic surface. As can be seen, the proportion of adherers with more than ten bacteria adhering per field was the dominant type. No particular bacterial isolates was significantly more able to adhere abiotic surfaces in both environmental isolates and collections strains.

3.5 *Hydrophobicity assay*

Employing the CHS classification scheme described by Schneider and Riley (1991), isolates with a % hydrophobicity greater than 70% were classified as highly hydrophobic and those with a hydrophobicity index less than 30% were classified as highly hydrophilic. The cell surface characteristics of isolates of *Pseudomonas aeruginosa* under examination are presented in Fig. 2. The isolates exhibited a variety of CSH, ranging from –7.83 - 42.22%. Using the bacterial adherence to hydrocarbons (BATH) method, only one of the 10 isolates from mineral water was

classified as hydrophobic. Majority of the isolates from environmental sources and collection strains were classified as highly hydrophilic.

3.6 Biofilm formation

P. aeruginosa is biofilm-forming, particularly when infecting the lungs of cystic fibrosis patients (Singh et al., 2000), but also in other infectious processes (Davey and O.Toole, 2000). Recently, Delissalde and Amabile-Cuevas (2004), using a simple method for quantifying biofilm formation, measured the production of biofilms by clinical isolates of *P. aeruginosa* and found that strains that produce biofilm are slightly more resistant to antibiotic when tested using planktonic methods than are non-biofilm formers.

In the present study, adherence to polystyrene microtiter wells by isolates from tap water, mineral water, artesian water, and the group of collections strains are showed in Fig. 3. A pronounced difference between environmental isolates and collections in the abilities to adhere was observed. Biofilm accumulated much more extensively in the environmental isolates.

3.7 ***Growth and biofilm development***

To quantitatively assess the biofilm development, growth in polystyrene microtiter wells of planktonic cells and biofilm formation in the same wells of ten environmental *Pseudomonas aeruginosa* isolates was monitored by using an assay base on viable plate counts (Fig. 4). The organisms grew well in TSB, reaching a

maximum number of approximately 10^9 CFU/ml at 36 h. The number of adherent bacteria was maximum (approximately 10^7 CFU/ml) at 12 h.

3.8 *Chlorine sensitivity*

3.8.1 Bactericidal effect

Chlorine is added to drinking water to reduce or eliminate microorganisms which can be present in water supplies. A number of bacteria have been shown to develop resistance to different agents used for the treatment of water, including chlorination (Ridgway and Olson, 1982; Pyle et al., 1994; Le Dantec et al., 2002). Ridgway and Olson (1982) reported that the most sensitive bacteria including *Pseudomonas* spp. are readily killed by chlorine concentrations of ≥ 1 mg/l. Recently, Howard and Inglis (2003) reported that *P. aeruginosa* responded to chlorine with a continued decline in viability over time. They also reported that tolerance to chlorine did not increase with subsequent exposure to chlorine, in contrast to the finding of Ridgway and Olson (1982) who showed that bacteria from chlorinated systems were more resistant than those from non-chlorinated systems.

In the current study, chlorine sensitivity tests revealed that 0.2 mg of chlorine per liter at a treatment time of 1 min kills 2 of the 3 strains from clinical strains, while only 3 of the 10 isolates from tap water and 4 out of 10 isolates each of the mineral water and artesian water, respectively, were killed at this concentration (Table 5). It is interestingly to note that three of ten, two of ten and one of three isolates from tap water, mineral water and clinical collection, respectively, were not killed by chlorine at 0.6 mg/l. This is particularly noteworthy because this chlorine concentration is 3 time the recommended level of ≥ 0.2 mg free chlorine per liter. Whether the higher

tolerance to chlorine of *Pseudomonas* aeruginosa isolates can be linked to greater virulence is not know.

3.8.2 Killing of Pseudomonas aeruginosa isolates by chlorine

Comparison of bacterial killing in the planktonic and biofilm states in response to treatment by chlorine is illustrated in Fig. 5. Time-kill curve of seven chlorine-resistant isolates from drinking water. Values represent the mean, and each mean value represent the average of three independent experiments on each isolate. The viability of planktonic cells (Fig. 5A) of *P. aeruginosa* isolates was rapidly reduced by 0.5 - 1.5 mg/l chlorine during the first 5 min, after which the rate of decrease in viability slowed. After 10 min exposure, there was a 100-fold reduction in viability of planktonic cells at 0.2 mg/ml chlorine concentration.

Biofilm bacteria were less susceptible to killing compared to planktonic cells is response to chlorine treatment (Fig. 5B). For the lowest concentration of chlorine (0.2 mg/l) tested, no killing of biofilm bacteria could be discerned even after prolonged exposure to the agent for 30 min. The same concentration of this biocide was sufficient to reduce 100-fold the viability of free-floating bacteria.

The mucoid exopolysaccharide produced by *P. aeruginosa* is a repeating polymer of mannuronic and glucoronic acid referred to as alginate (Dunne and Buckmire, 1985). Alginate slime forms the matrix of the *Pseudomonas* biofilm which anchors the cells to their environmental and, in medical situation, it protects the bacteria from the host defenses such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement. Biofilm mucoid strains of *P. aeruginosa* are also less susceptible to antibiotics than their planktonic counterparts.

Mucoid strains of *P. aeruginosa* are most often isolated from patients with cystic fibrosis and they are usually found in post mortem lung tissue from such individuals (Zielinski et al., 1992).

3.9 Antibiotic susceptibility

The antibiotic susceptibility test was performed by the standard disc diffusion method. According to our results, a highest prevalence of resistance among the total number of *Pseudomonas aeruginosa* isolates was shown for chloramphenicol, gentamicin and trimethoprim-sulfamethoxazole. A low prevalence of cefotaxime resistance was detected in environmental isolates (Table 6).

P. aeruginosa is inherently resistant to many antimicrobial agents mainly due to the synergy between multi-drug efflux systems or a type 1AmpCβ-lactamase and low outer membrane permeability (Livermore, 2002). According to Corona-Nakamura et al. (2001), although identical resistance profiles do not indicate an identical genetic source, a multiresistant pattern or an unexpected change in the laboratory reports can be the first clue that draws the attention of the physician assigned to a clinical area.

In our study, prevalence of multiple-antibiotic resistance among *Pseudomonas aeruginosa* strains isolated from tap water, mineral water and artesian water and collections strains is presented in Table 7. Resistance to three or more antibiotic was frequently observed among isolates from environmental and clinical strains.

Although all drinking water isolates were susceptible to aztreonam, cepepime, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin-tazobactam, and

polymyxin, the *Pseudomonas aeruginosa* isolates have demonstrated resistance to one or more antibiotics. The increasing prevalence of resistance in the isolates from environmental sources may have important therapeutic implications. More restrictive policies on the use of antibiotics in human and animals may improve the current situation.

3.10 *Serum sensitivity*

Of the 35 *P. aeruginosa* strains (including clinical and type strains ATCC) examinated, only five isolates from mineral water were classified as serum-sensitive (Table 8). In contrast, all the samples isolates from artesian water were resistant to 50% human serum.

In one study on *P. aeruginosa* (Yong and Armstrong, 1972) , 78% of saprophytic strains and 91% of isolates from bacteremic human infections were found to be resistant to the bactericidal activity of human serum. Schiller and Hatch (1983) have reported that slime-producing strains were generally more serum-sensitive than non-slime producers, although serum sensitivity was not related to slime production per se. According to these authors the clinical isolation site appeared to be the predominant determinant of a strain's sensitive.

3.11 Association between virulence markers

Analyses of the inter-relationship between markers of virulence were examined using Spearman correlation coefficients (Table 9). A positive correlation was observed among hemolysis, cytotoxicity, and adherence to glass as well as

between serum sensitivity and adherence to Vero cells. The adherence to human epithelial cells was positively correlated with hemolysis, cytotoxicity and adherence to glass, whereas adherence to silicon was positively correlated with adherence to PET.

The results of this study show that a notable fraction of *P. aeruginosa* isolates from drinking water were able to elaborate virulence factors, and the incidence of virulence properties was not statistically different among the three sources. In addition, there was a strong and statistically correlation between adherence and cytotoxicity among the totality of the isolates, indicating that expressing both virulence properties may be essential for *P. aeruginosa* during infections. Rajan et al. (2000), recently demonstrated that only fully virulent *P. aeruginosa* capable of coordinately expressing both adhesin and cytotoxins were able to induce apoptosis in respiratory epithelial cells.

The results presented here on the virulence profiles of *P. aeruginosa* isolates emphasizes the need of more extensive study about the virulence of this bacterium isolated from environmental sources. In this case, research about the virulence properties of this bacterium by toxic assays on animal should be explored. Still more interesting would be toxicity assays on immunodeficient animals and collect information on people drinking waters containing *P. aeruginosa.*

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Table 1. Hemolysis and hemagglutination reaction of *Pseudomonas aeruginosa* strains isolated from tap water, mineral water and artesian water[a].

Sources		No. of isolates showing:	
	n	Hemolysis	Hemagglutination
Tap water	10	10	3
Mineral water	10	10	6
Artesian water	10	10	0
Clinical strains	3	3	0
ATCC strains	2	2	1

[a] Sheep blood

Table 2. Cytotoxicity of *Pseudomonas aeruginosa* strains isolated from tap water, mineral water and artesian water to Vero cells.

Supernatant titers	No. of isolates showing cytotoxicity				
	Tap water (n=10)	Mineral water (n=10)	Artesian water (n=10)		
				Clinical strains (n=3)	ATCC strains (n=2)
1/10	2	4	3	1	0
1/100	5	4	3	0	2
1/1000	2	2	0	2	0
1/10000	1	0	2	0	0
Nontoxic	0	0	2	0	0

Table 3. Adhesion of *Pseudomonas aeruginosa* strains isolated from tap water, mineral water, and artesian well water to epithelial cells.

Bacteria per cell	No. of isolates					
	Tap water (n=10)		Mineral water (n=10)		Artesian water (n=10)	
	Vero[a]	BEC[b]	Vero	BEC	Vero	BEC
0-5	7	0	9	0	2	0
>5-10	3	1	1	0	8	0
>10-100	0	8	0	10	0	8
>100	0	1	0	0	0	2

Bacteria per cell	No. of isolates			
	Clinical strains (n=3)		ATCC strains (n=2)	
	Vero	BEC	Vero	BEC
0-5	1	0	0	0
>5-10	1	0	1	0
>10-100	1	2	1	2
>100	0	1	0	0

[a] Vero cells
[b] BEC = human buccal epithelial cell.

Table 4. Adhesion of *Pseudomonas aeruginosa* strains isolated from tap water, mineral water and artesian water to abiotic surfaces.

Bacteria per field	No. of isolates								
	Tap water (n=10)			Mineral water (n=10)			Artesian water (n=10)		
	Glass	Silicon	PET*	Glass	Silicon	PET	Glass	Silicon	PET
0-5	2	1	0	3	4	7	1	6	6
>5-10	2	1	0	1	3	1	3	2	0
>10-100	5	7	8	6	2	2	6	2	4
>100	1	1	2	0	1	0	0	0	0

Bacteria per field	No. of isolates					
	Clinical strains (n=3)			ATCC strains (n=2)		
	Glass	Silicon	PET	Glass	Silicon	PET
0-5	0	0	2	0	1	1
>5-10	1	0	0	0	1	1
>10-100	2	3	1	2	0	0
>100	0	0	0	0	0	0

* Polyethylene terephthalate

Table 5. Resistance patterns of *Pseudomonas aeruginosa* isolated from tap water, mineral water, and artesian water to chlorine[a].

Chlorine concentrations (mg/l)	No. of isolates with resistance status			
	Tap water (n=10)	Mineral water (n=10)	Artesian water (n=10)	Clinical strains (n=3)
1.0	3	0	0	1
0.8	3	1	0	1
0.6	3	2	0	1
0.4	3	2	3	1
0.2	7	6	6	1
0.1	8	9	9	3

[a] After a 1-min contact.

Table 6. Prevalence of antibiotic resistance among *Pseudomonas aeruginosa* strains isolated tap water, mineral water and artesian water.

Antibiotics	No. of isolates with resistance status				
	Tap water (n=10)	Mineral water (n=10)	Artesian water (n=10)	Clinical strains (n=3)	ATCC strains (n=2)
Monobactam					
Aztreonam	0	0	0	1	0
Cephems					
Cefepime	0	0	0	3	0
Cefotaxime	3	1	0	3	0
Ceftazidime	0	0	0	3	0
Phenicol					
Chloramphenicol	9	8	6	3	2
Fluoroquinolone					
Ciprofloxacin	0	0	0	2	0
Aminoglycoside					
Gentamicin	3	3	6	3	0
Carbapenems					
Imipenem	0	0	0	3	0
Meropenem	0	0	0	3	0
β-lactams					
Piperacillin-Tazobactam	0	0	0	2	0
Folate pathway inhibitor					
Trimethoprim-Sulfamethoxazole	10	8	10	3	2
Polymyxin					
Polymyxin B	0	0	0	0	0

Table 7. Prevalence of multiple-antibiotic resistance among *Pseudomonas aeruginosa* strains isolated from tap water, mineral water and artesian water.

	No. of isolates				
	Tap water (n=10)	Mineral water (n=10)	Artesian water (n=10)		
				Clinical strains (n=3)	ATTC strains (n=2)
Sensitivity	0	0	0	0	0
Resistance to one antibiotic	1	3	2	0	0
Resistance to two antibiotic	4	4	4	0	2
Resistance to three or more antibiotic	5	3	4	3	0

Table 8. Serum sensitivity of *Pseudomonas aeruginosa* isolated from tap water, mineral water, and artesian water.

Source	*n*	No. of isolates		
		Sensitive	Intermediate sensitive	Resistant
Tap water	10	0	3	7
Mineral water	10	5	4	1
Artesian water	10	0	0	10
Clinical strains	3	0	0	3
ATCC strains	2	0	1	1

Sensitive = >90% kill at ≤6.25% HS
Intermediate = >90% kill at 12.5%-25% HS
Resistant = ≤90% kill at ≥50% HS

Table 9. Spearman correlation coefficients among markers of virulence of *Pseudomonas aeruginosa* isolates from tap water, mineral water, and artesian water.

Markers	Correlation coefficient								
Hemolysis	0.56								
Hemagglutination	-0.39	0.52							
Citotoxicity	0.34	0.95*	0.73						
Multiple-antibiotic resistance	0.71	0.78	0.16	0.74					
Adherence to glass	0.40	0.96*	0.67	0.96*	0.63				
Adherence to silicon	0.51	0.69	0.34	0.77	0.82	0.70			
Adherence to PET	0.48	0.41	0.10	0.50	0.68	0.43	0.94*		
Adherence to Vero cell	0.89*	0.31	0.62	0.00	0.40	0.12	0.06	0.04	
Adherence to BEC	0.58	1.00*	0.52	0.96*	0.80	0.96*	0.76	0.49	0.29
	Serum-resistant	Hemolysis	Hemagglutination	Citotoxicity	Multiple-antibiotic resistance	Adherence to glass	Adherence to silicon	Adherence to PET	Adherence to Vero cells

Significance level: *P <0,05.

LEGEND FOR FIGURES

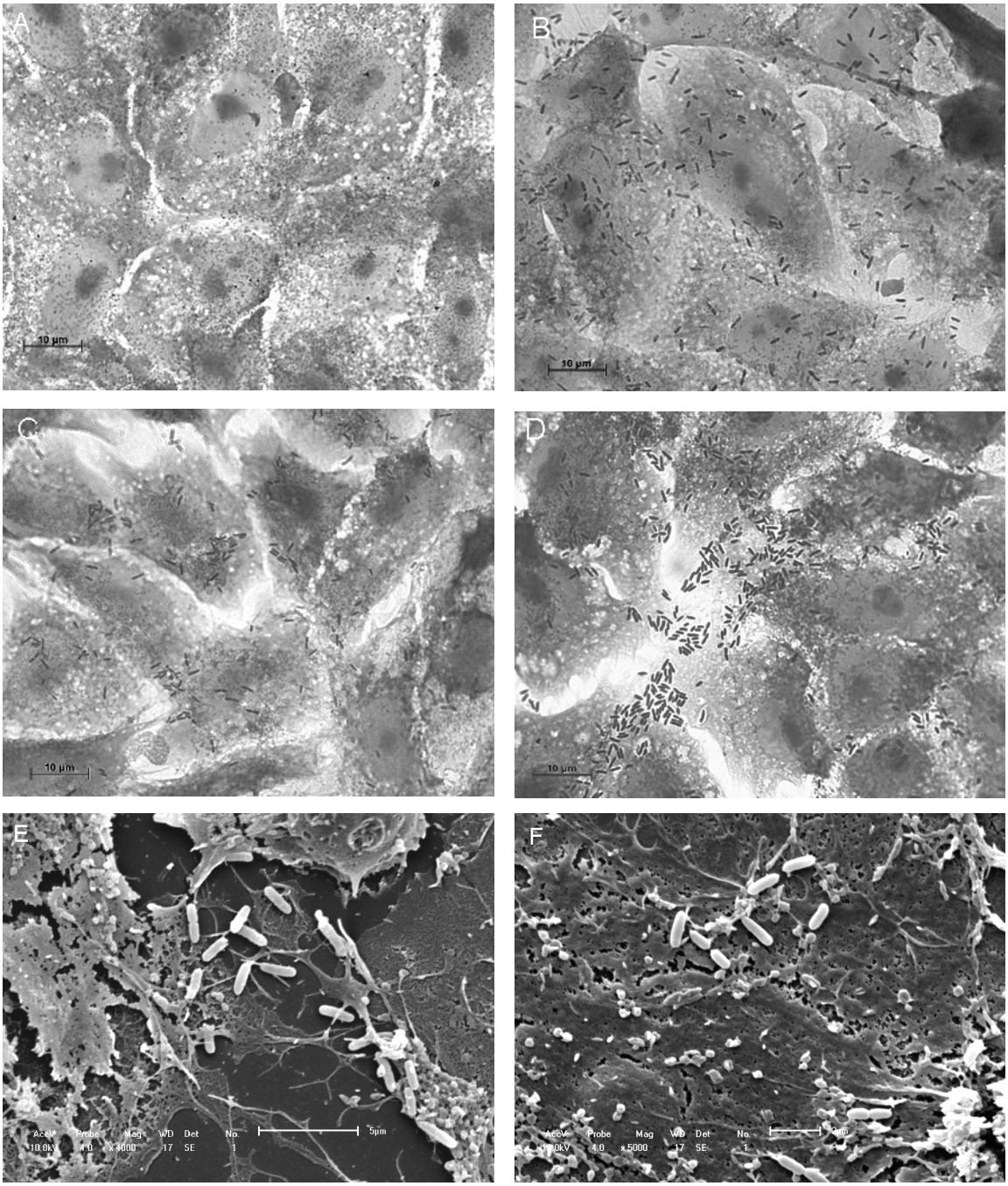
Figure 1. Light microscopy (**A-D**) and scanning electron micrographs (**E-F**) showing the types of *Pseudomonas aeruginosa* adherence patterns: **A**, Vero cells alone; **B**, diffuse adhesion; **C**, localized adhesion (small clusters), and **D**, aggregative adhesion (huge clusters of adhering bacteria). **E**, subconfluent Vero cell images show the basal extension of connections with the bacteria. **F**, confluent Vero cell images show the general lack of extensions to the bacteria, with the exception of few connections between cells.

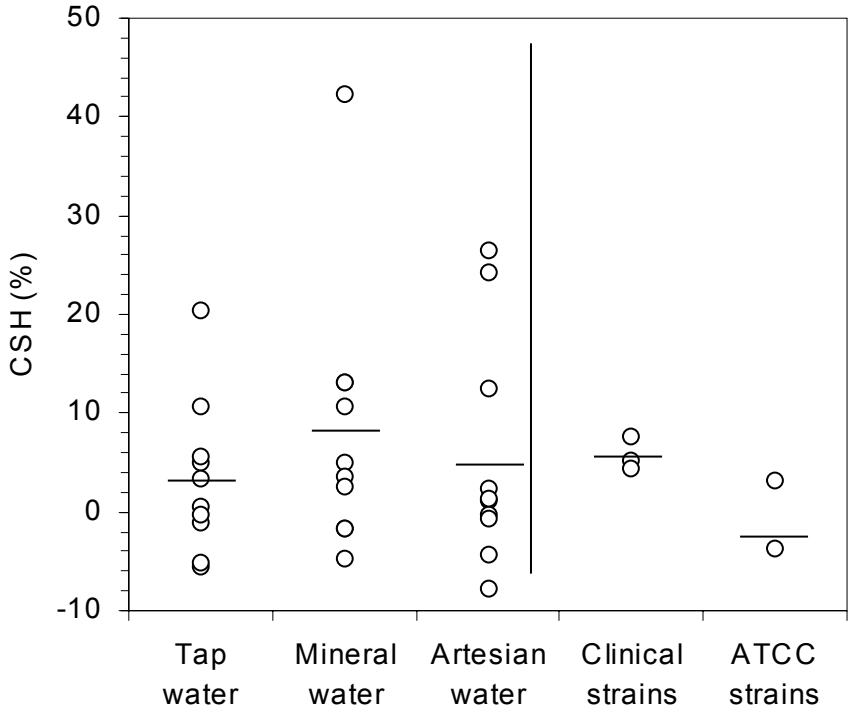
Figure 2. CSH (%) of different strains of *Pseudomonas aeruginosa* isolates from tap water, mineral water, artesian water, and the group of collections strains (clinical and type strains), as measured by adhesion to p-xylene. The dots indicate the average of triplicate determination. The bar indicate the median of the plotted values.

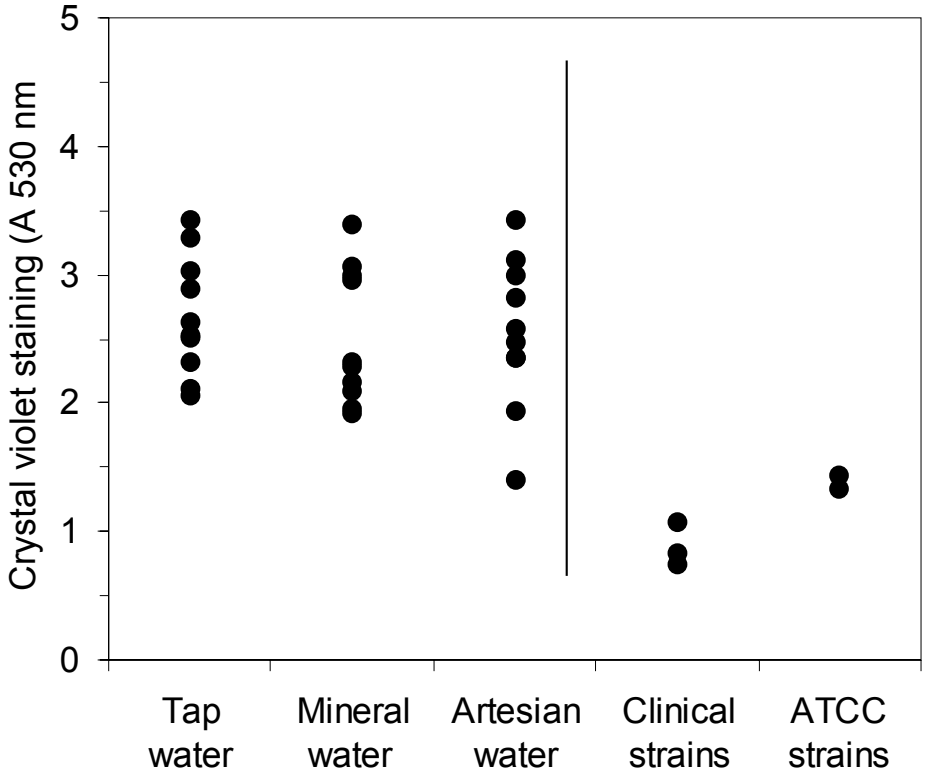
Figure 3. Adherence to polystyrene microtiter wells by *Pseudomonas aeruginosa* isolates from tap water, mineral water, artesian water, and the group of collections strains (clinical and type strains) after incubation of 12 h at 37°C. The dots indicate the average of triplicate determination.

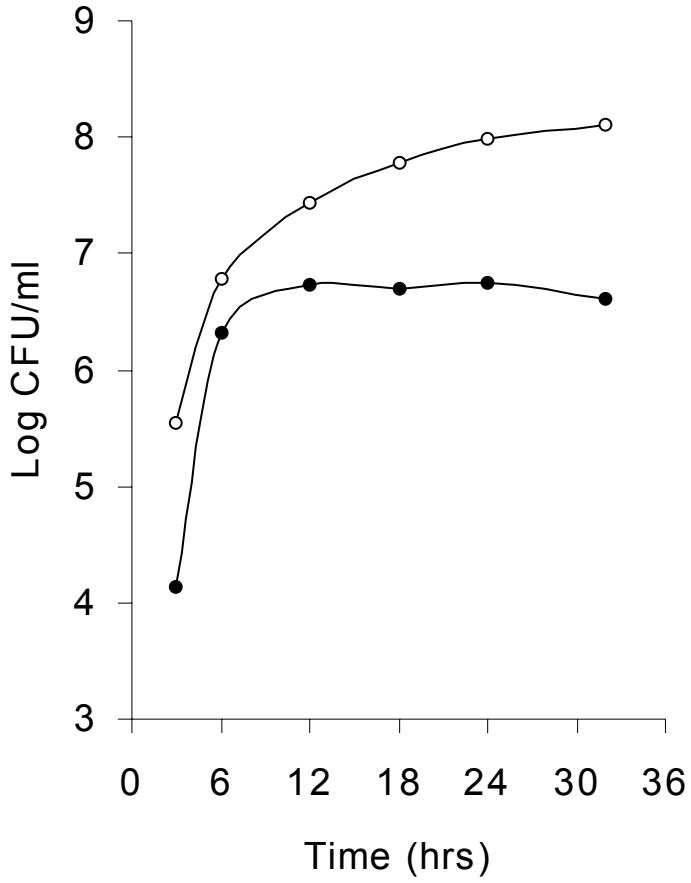
Figure 4. Biofilm formation by environmental strains. Growth in polystyrene microtiter wells of planktonic cells (open circles). Biofilm formation in the same wells is show as filled circles. Values represent the mean. Each mean value represent the average of three independent experiments on each isolate.

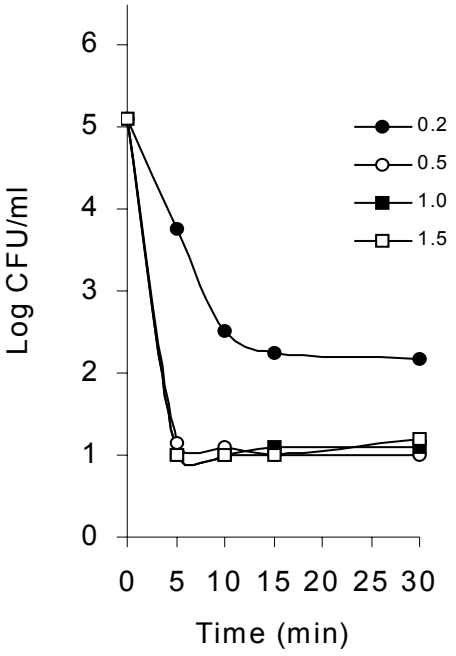
Figure 5. Killing of *Pseudomonas aeruginosa* isolates by chlorine. (A) Planktonic cells and (B) biofilm formed in the same wells were treated with chlorine and then plated for colony counting. Time-kill curve of seven chlorine-resistant isolates drinking water tested as described in Materials and Methods. Values represent the mean. Each mean value represent the average of three independent experiments on each isolate.

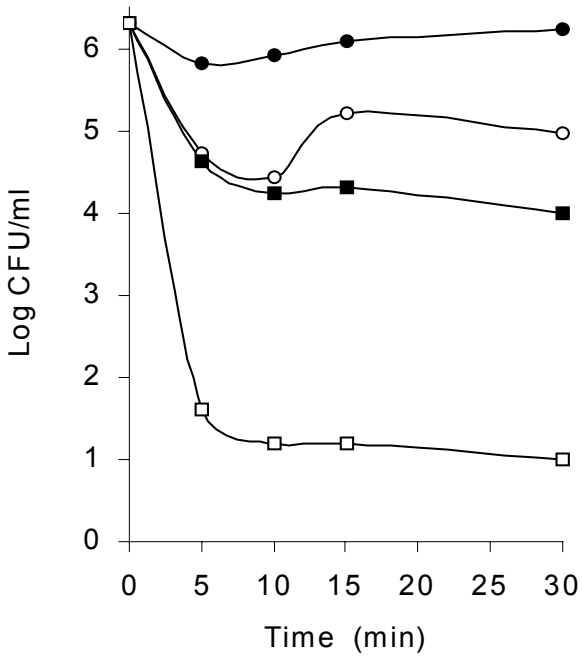

A
10 µm
B
10 µm
C
10 µm
D
10 µm
E
Acc.V Probe Mag WD Det No.
10.0kV 4.0 x5000 17 SE 1
5µm
F
Acc.V Probe Mag WD Det No.
10.0kV 4.0 x5000 17 SE 1
2µm


50
40
30
20
10
0
-10
CSH (%)
Tap water
Mineral water
Artesian water
Clinical strains
ATCC strains


Crystal violet staining (A 530 nm
5
4
3
2
1
0
Tap water
Mineral water
Artesian water
Clinical strains
ATCC strains


9
8
7
6
5
4
3
Log CFU/ml
0
6
12
18
24
30
36
Time (hrs)


Log CFU/ml
Time (min)
0.2
0.5
1.0
1.5


Log CFU/ml
Time (min)

ANEXOS

Tabela 1. Características físico-químicas da água de solução alternativa de abastecimento.

Parâmetros	Valores Médios (Faixa)
pH	7.15 (6 – 8.6)
Nitrogênio Total (mg l^{-1})	2.4 (0.5 – 3.8)
Carbono Orgânico Total (mg l^{-1})	1.47 (0.4 – 3.4)
Carbono Total (mg l^{-1})	9.74 (7.4 – 14.5)
Carbono Inorgânico (mg l^{-1})	8.3 (6 – 13)
Fósforo (μg l^{-1})	71.4 (44.8 – 92.3)

Tabela 2. Qualidade microbiológica de água potável de solução alternativa de abastecimento.

Bactérias Indicadoras	Número e porcentagem de amostras positivas			
	Clorada (n=23)		Não-Clorada (n=47)	
	Poço (n=08)	Torneira (n=15)	Poço (n=26)	Torneira (n=21)
Coliformes Totais	0	03 (20.0)	03 (11.5)	09 (42.8)
Coliformes Termotolerantes	0	02 (13.3)	01 (3.80)	03 (14.3)
Escherichia coli	0	01 (6.70)	0	01 (4.80)
Enterococos	0	01 (6.70)	02 (7.70)	04 (19.0)
Pseudomonas aeruginosa	03 (37.5)	08 (53.3)	12 (46.1)	08 (38.0)
Staphylococcus aureus	0	03 (20.0)	08 (30.8)	01 (4.80)
Bactérias Heterotróficas				
<1	03 (37.5)	02 (13.3)	03 (11.5)	0
1-500	03 (37.5)	10 (66.7)	19 (73.0)	16 (76.2)
501 – 1000	02 (25.0)	03 (20.0)	01 (3.80)	04 (19.0)
1001 – 100.000	0	0	03 (11.5)	01 (4.80)
Amostras Inaceitáveis				
- HPC	03 (37.5)	08 (53.3)	19 (73.0)	16 (76.2)
+ HPC *	05 (62.5)	09 (60.0)	19 (73.0)	17 (81.0)

* HPC > 500UFC/ml.

Tabela 3. Número de amostras inaceitáveis em relação à concentração de cloro (mg l^{-1}).

Cloro (Faixa)	Número de amostras inaceitáveis [1]			
	- HPC [2]		+ HPC	
	Poço	Torneira	Poço	Torneira
<0,2	12/34	13/36	13 /34	13/36
≥0,2 – 0,4	01/34	02/36	01/34	02/36
>0,4	01/34	02/36	02/34	02/36

[1] – Amostras positivas para ao menos um indicador ou bactéria patogênica.

CONCLUSÕES

A qualidade bacteriológica de água de torneira do sistema municipal de distribuição é superior a qualidade de água de galões de 20 litros em dispensadores e amostras coletadas de novos galões antes da instalação nos dispensadores.

Há uma grande necessidade de um aumento na melhora para o sistema industrial de água engarrafada. Melhores esforços são necessários para eliminar bactérias planctônicas e biofilme, ambos fontes de contaminação que podem concentrar patógenos oportunistas como *Pseudomonas aeruginosa.* Em adição, freqüente limpeza dos dispensadores pode ajudar a eliminar vários contaminantes da água e diminuir a possibilidade de doenças veiculadas pela água.

Para sistemas de distribuição de água, a enumeração de *Pseudomonas* deve ser realizada periodicamente, em adição aos dados coletados na rotina desses sistemas de distribuição.

Uma notável fração de *P. aeruginosa* isoladas de água potável demonstraram possuir fatores de virulência. Os resultados apresentados sobre perfil de virulência desses isolados enfatizam a necessidade de estudos sobre a virulência de bactérias isoladas de fontes ambientais.

Todos os isolados de *P. aeruginosa* demonstraram resistência a um ou mais antibióticos, e uma resistência importante foi ao antibiótico cefotaxima, uma Cefalosporina de 3ª geração.