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FACULTAD DE CIENCIAS DE LA SALUD

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LICENCIATURA EN TECNOLOGÍA MÉDICA

TRABAJO DE GRADUACIÓN:

EVALUACIÓN AMBIENTAL DE LA PRESENCIA DE LEGIONELLA PNEUMOPHILA EN SISTEMAS DE AIRE ACONDICIONADO EN HOSPITALES

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Quiero agradecer primeramente a Dios por darme la fuerza, sabiduría y entendimiento a lo largo de este camino.

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"¡Los sueños de la gente... nunca terminan!"

Marshall D. Teach (Kurohige)

José Flores

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Karen Powell

DECLARACIÓN JURADA

Yo, **José Luis Flores Hughes,** con cédula de identidad personal número **3-753-920**, estudiante graduando del programa/carrera de **Licenciatura en Tecnología Médica,** declaro bajo la gravedad del juramento que el material que aparece en este trabajo de graduación, en la opción **revisión bibliográfica,** es de mi producción intelectual, en razón de lo cual exonero a la Universidad Latina de Panamá de cualquier responsabilidad relacionada con este aspecto.

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Cédula: _____

Yo, **Karen Michelle Powell Morán,** con cédula de identidad personal número **3-752-2368**, estudiante graduando del programa/carrera de **Licenciatura en Tecnología Médica,** declaro bajo la gravedad del juramento que el material que aparece en este trabajo de graduación, en la opción **revisión bibliográfica,** es de mi producción intelectual, en razón de lo cual exonero a la Universidad Latina de Panamá de cualquier responsabilidad relacionada con este aspecto.

Como constancia, firmo la presente declaración el día _____ del mes de

_____ del año _____.

Firma del estudiante: _____

Cédula: _____

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RESUMEN

Antecedentes: Legionella pneumophila es una bacteria que se desarrolla en ambientes acuáticos. Puede proliferar en sistemas de agua y climatización, especialmente cuando existen condiciones como estancamiento del agua, formación de biopelículas y mantenimiento inadecuado. Se ha reconocido que los sistemas de aire acondicionado y torres de enfriamiento son posibles fuentes de transmisión mediante aerosoles contaminados. Esto se convierte en un problema relevante para la salud pública.

Objetivo: Analizar la evidencia científica disponible sobre la presencia de Legionella pneumophila en los sistemas de aire acondicionado hospitalarios. Identificar los factores que favorecen su proliferación. Evaluar las estrategias de prevención y control descritas en la literatura. Además, determinar el papel de estos sistemas como reservorios de la bacteria y su relación con las infecciones nosocomiales.

Métodos: Se llevó a cabo una búsqueda sistemática en las bases de datos Scopus, PubMed, ScienceDirect y repositorios institucionales. Se incluyeron estudios publicados entre 2018 y 2025 en inglés y español, utilizando el método PRISMA para hacer una selección de los artículos más transparente y ordenada.

Resultados: La revisión mostró que el crecimiento de Legionella pneumophila está muy ligado al agua estancada, la creación de biopelículas, temperaturas entre 25 °C y 45 °C, bajos niveles de desinfectantes y problemas en el mantenimiento. Se observó que las técnicas moleculares como la PCR y la qPCR presentan una mayor sensibilidad que los métodos de cultivo convencionales. Entre las estrategias de control más eficaces destacan la desinfección continua con monocloramina y la instalación de grifos de flujo temporal (TFT).

Conclusiones: El análisis crítico permitió concluir que el problema no radica únicamente en el aire acondicionado, sino en todo el sistema de climatización. La falta de mantenimiento y la contaminación en las torres de enfriamiento y sus tuberías favorecen la formación y dispersión de aerosoles, que pueden ser liberados a través de las salidas del aire acondicionado.

Palabras clave: Legionella pneumophila, aire acondicionado, torres de enfriamiento, hospitales, monitoreo ambiental.

ABSTRACT

Background: Legionella pneumophila is a bacterium that thrives in aquatic environments and can proliferate in water and air conditioning systems, especially under situations such as stagnant water, biofilm formation, and inadequate maintenance. Air conditioning systems and cooling towers have been recognized as potential sources of transmission via contaminated aerosols, making it a significant public health concern.

Objective: To analyze the available scientific evidence on the presence of Legionella pneumophila in hospital air conditioning systems. Identify factors that promote its proliferation. Evaluate prevention and control strategies described in the literature. Finally, determine the role of these systems as reservoirs of the bacterium and their relationship to nosocomial infections.

Methods: A systematic search was conducted in the Scopus, PubMed, and ScienceDirect databases, as well as institutional repositories. Studies published between 2018 and 2025 in English and Spanish were included, using the PRISMA method to select the most transparent and well-organized articles. Results: The review shows that Legionella pneumophila proliferation is closely related to stagnant water, biofilm formation, temperatures between 25 °C and 45 °C, low disinfectant levels, and inadequate maintenance. Molecular techniques such as PCR and qPCR were found to be more sensitive than conventional culture methods. Among the most effective control strategies are continuous disinfection with monochloramine and the installation of time-of-flow taps (TFTs).

Conclusions: The critical analysis concluded that the problem lies not only in the air conditioning system but in the entire HVAC system. Lack of maintenance and contamination in cooling towers and their piping promote the formation and dispersion of aerosols, which can be released through the air conditioning vents.

Keywords: Legionella pneumophila, air conditioning, cooling towers, hospitals, environmental monitoring.

INTRODUCCIÓN

La legionelosis es una enfermedad infecciosa causada principalmente por la bacteria *Legionella pneumophila*, un microorganismo gramnegativo que habita de forma natural en ambientes acuáticos y que puede proliferar en sistemas artificiales de distribución de agua y climatización. Esta enfermedad adquirió relevancia mundial tras el brote epidémico ocurrido en 1976 durante la convención de la Legión Americana celebrada en un hotel de Filadelfia, Estados Unidos, donde numerosos asistentes desarrollaron neumonía grave. Cinco meses después del evento, se logró identificar el agente etiológico responsable, denominado Legionella pneumophila, bacteria que hasta entonces no había sido reconocida como causante de enfermedad en humanos (Vaqué & Martínez, 2002).

Posteriormente, las investigaciones lideradas por Fliermans permitieron demostrar que Legionella se encontraba ampliamente distribuida en ambientes acuáticos naturales y artificiales, particularmente en aguas templadas y sistemas de refrigeración. Estos estudios evidenciaron que la transmisión de la bacteria podía producirse mediante la inhalación de gotas de agua aerosolizadas provenientes de sistemas de aire acondicionado contaminados, constituyendo un importante avance en la comprensión de la epidemiología de la enfermedad (Ferrer, 2023).

Debido a su relevancia para la salud pública, organismos internacionales como la Organización Mundial de la Salud (OMS) han incorporado a Legionella dentro de los microorganismos de importancia para la calidad del agua potable. Destacan la necesidad de implementar programas de vigilancia, monitoreo y control en sistemas de abastecimiento de agua y equipos de climatización (OMS, 2022). De igual manera, los Centros para el Control y la Prevención de Enfermedades (CDC, 2017) señalan que los sistemas de calefacción, ventilación y aire acondicionado (HVAC) constituyen un componente esencial en los programas de prevención y control de infecciones, especialmente en instalaciones hospitalarias, donde su correcto funcionamiento contribuye a mantener condiciones ambientales seguras y a reducir la propagación de agentes patógenos.

En este contexto, resulta fundamental analizar la evidencia científica disponible sobre las medidas de prevención y control de Legionella pneumophila en sistemas de agua y climatización, con el propósito de contribuir a la reducción del riesgo de transmisión, fortalecer las estrategias de vigilancia epidemiológica y promover entornos más seguros para la población y los servicios de salud.

CAPÍTULO 1: PLANTEAMIENTO DEL PROBLEMA

1.1 NECESIDAD DEL ESTUDIO

A nivel global, la seguridad ambiental en los centros de salud ha cobrado una

Relevancia crítica debido a la complejidad de las infraestructuras de climatización. La bacteria Legionella pneumophila representa uno de los mayores desafíos para la ingeniería sanitaria, ya que posee la capacidad de formar biofilms en sistemas que manejan agua y aire, tales como torres de enfriamiento y bandejas de condensación. La problemática se origina cuando el mantenimiento preventivo es deficiente, permitiendo que el estancamiento del agua, temperaturas inferiores a 50 °C y la acumulación de sedimentos favorezcan la proliferación bacteriana y, como consecuencia, resultados caóticos para pacientes inmunocomprometidos (Hart CA, 1991).

Este riesgo es alarmante debido a que la liberación de aerosoles a través de los ductos de aire acondicionado puede provocar brotes de legionelosis, una enfermedad cuya letalidad en pacientes inmunocomprometidos es significativamente alta. A pesar de que la normativa internacional exige controles estrictos, en muchos entornos locales la vigilancia microbiológica de estos sistemas es escasa o inexistente, lo que genera una falla persistente en la bioseguridad ocupacional y clínica. Partiendo de esta situación, se plantea la siguiente pregunta general: ¿Cuáles son los factores determinantes en la proliferación de Legionella pneumophila en los sistemas de aire acondicionado hospitalarios y qué estrategias de control basadas en evidencia permiten mitigar su impacto en la salud pública?

1.2 OBJETIVOS

1.2.1 OBJETIVO GENERAL

- Analizar la evidencia científica disponible sobre la presencia de Legionella pneumophila en sistemas de aire acondicionado.

1.2.2 OBJETIVOS ESPECÍFICOS

- Identificar los factores ambientales, estructurales y de mantenimiento que favorecen la proliferación de la Legionella pneumophila.
- Determinar la eficacia de las estrategias preventivas de los hospitales reportadas en la literatura científica.
- Evaluar el papel de los sistemas de aire acondicionado como reservorios de Legionella pneumophila y su implicación en la prevención de infecciones nosocomiales en entornos hospitalarios.

1.3 JUSTIFICACIÓN

Los centros hospitalarios albergan a poblaciones con sistemas inmunológicos comprometidos, para quienes la exposición a la Legionella pneumophila puede resultar fatal. Los resultados de este estudio beneficiarán directamente a los departamentos de ingeniería clínica y medicina preventiva, proporcionando una base documental sólida para la gestión de riesgos.

En cuanto a sus implicaciones prácticas, ofrece criterios claros para la toma de decisiones sobre qué sistemas de desinfección son más costoefectivos y seguros. El valor teórico de la investigación radica en la integración de conocimientos dispersos sobre la ecología bacteriana en ambientes artificiales, aportando una síntesis actualizada que profundiza en la relación entre el diseño de los sistemas HVAC (calefacción, ventilación y aire acondicionado) y la persistencia de biofilms. Finalmente, la utilidad del método se muestra en la creación de un marco de evaluación que puede ser un ejemplo para investigaciones futuras o para desarrollar herramientas de auditoría interna. Esto ayuda a aplicar mejor los protocolos de mantenimiento a largo plazo en las instituciones de salud.

CAPÍTULO 2 MARCO TEÓRICO

2.1 ANTECEDENTES HISTÓRICOS

La legionelosis fue reconocida por primera vez en un importante brote epidémico de neumonía que afectó a los asistentes a la convención de la Legión Americana, celebrada en un hotel de Filadelfia en 1976. Cinco meses después del brote se identificó el agente etiológico Legionella pneumophila, un bacilo que no había sido reconocido hasta entonces como causa de enfermedad humana. El examen de

especímenes, recogidos anteriormente, permitió asociar dicho agente con brotes epidémicos ocurridos cerca de treinta años antes (Vaqué & Martínez, 2002). Este estudio marcó el inicio de múltiples investigaciones orientadas a comprender los mecanismos de transmisión de esta bacteria, estableciendo su relación con sistemas de aire acondicionado contaminados.

Tras el descubrimiento de McDade, el Dr. Carl Fliermans descubrió que los lípidos de la Legionella se parecían a los de las bacterias que había encontrado en las regiones templadas del Parque Nacional de Yellowstone. Estas bacterias tienden a vivir en temperaturas templadas y en los biofilms, asociadas con algunas especies de algas. Posteriormente, Fliermans empezó a estudiar otros hábitats acuáticos y encontró que esta bacteria reside en las aguas templadas naturales en todos los Estados Unidos y, sobre todo, en las torres de refrigeración de aire acondicionado; esto permitió descubrir que el bacilo de la legionelosis se había transmitido por el sistema de aire acondicionado, en las gotas de agua aerosolizadas (Ferrer, 2023).

Posteriormente, organismos internacionales como la Organización Mundial de la Salud (OMS, 2022) incorporaron a Legionella dentro de los patógenos de importancia en la calidad del agua potable, destacando la necesidad de establecer programas de monitoreo y control en sistemas de agua y climatización. Según la Guía de control de infecciones sobre calefacción, ventilación y aire acondicionado (HVAC) para jefes de enfermería y médicos de los Centros para el Control y la Prevención de Enfermedades (CDC, 2017), un sistema de calefacción, ventilación y aire acondicionado es una parte integral de los protocolos de control de infecciones de un hospital. El sistema de HVAC es responsable de templar el aire, ajustar la humedad relativa en un espacio, establecer relaciones de presurización entre lugares, filtrar y diluir el aire recirculado a través del edificio, ventilarlo y eliminar los contaminantes de los ambientes. En el estudio titulado “Enumeración de Legionela Pneumophila en agua de las torres de enfriamientos de las instalaciones en el Área Metropolitana De La Cuidad De Panamá”, se implementó la prueba Legiolert (un ensayo basado en cultivo para L. pneumophila basado en el número más probable [MPN]) en 20 instalaciones del área metropolitana de la Ciudad de Panamá con un total de 98 torres de enfriamiento muestreadas; se evidencio evidenció, a través del análisis de los parámetros fisicoquímicos, que factores como la temperatura, el pH y la concentración de desinfectantes influyen directamente en el crecimiento de la bacteria, destacando la necesidad de una adecuada gestión y mantenimiento de las torres de enfriamiento (Morris, 2021).

2.2 MAGNITUD DEL PROBLEMA

Desde el primer brote identificado en 1976 en la convención de la Legión Americana en Filadelfia, persisten lagunas cruciales en la investigación sobre medidas de prevención y control que, a pesar de avances sustanciales en la comprensión de la transmisión de la legionella, obstaculizan los esfuerzos por reducir el aumento recurrente de casos. Los brotes mundiales de legionelosis registrados en 2024 en

Australia (114 casos y 2 defunciones), Italia (53 casos y 4 defunciones), EE. UU. (30 casos y 2 defunciones), Reino Unido (10 casos y 3 muertes), Nueva Zelanda (108 casos) ponen de relieve la necesidad imperativa de medidas preventivas entre los profesionales de la salud y la comunidad. (Pareek A et al., 2024).

En República de Panamá, el estudio realizado por Morris (2021) evidenció la presencia de Legionella pneumophila significativa en infraestructuras críticas, detectándose en más del 50 % de las torres de enfriamiento analizadas en la Ciudad de Panamá, tanto en la estación lluviosa (56.6 %) como seca (51.1 %). Aunque la frecuencia de detección ambiental se elevó, los casos clínicos reportados de legionelosis parecen estar subregistrados, lo que podría reflejar limitaciones en los sistemas de vigilancia epidemiológica y diagnóstico de la enfermedad.

Según la Organización Mundial de la Salud (2022), la incidencia identificada de la legionelosis varía considerablemente según el nivel de vigilancia y notificación. Dado que muchos países carecen de métodos adecuados para diagnosticar la infección o de sistemas de vigilancia suficientes, se desconoce la tasa de incidencia. En Europa, Australia y Estados Unidos se detectan entre 10 y 15 casos por millón de habitantes al año.

Entre los factores de riesgo para la legionelosis se incluyen el tabaquismo, antecedentes de consumo excesivo de alcohol, enfermedades pulmonares, inmunosupresión y enfermedades respiratorias o renales crónicas.

En el ámbito intrahospitalario, la neumonía nosocomial por Legionella incluye factores como cirugía reciente, intubación (intubación traqueal), ventilación mecánica, aspiración, presencia de sondas nasogástricas y uso de equipos de terapia respiratoria; los más susceptibles a esto son aquellos pacientes inmunocomprometidos, como receptores de trasplantes de órganos, con cáncer y quienes reciben tratamiento con corticosteroides.

Se sospecha que los bioaerosoles de las unidades de refrigeración aire-agua suelen causar brotes de legionelosis adquirida en la comunidad. Aunque las infecciones *por Legionella* se pueden atribuir mayoritariamente a fuentes de emisiones, existe incertidumbre sobre la liberación y distribución de bacterias en el aire, la aparición de la forma virulenta respirable y el nivel de concentración infecciosa. Para prevenir y controlar la contaminación *por Legionella* de las unidades de refrigeración aire-agua, las acciones de mantenimiento deben centrarse en procedimientos de limpieza de bajas emisiones combinados con mediciones de control de muestras de agua y aire. Los procedimientos que permiten la detección rápida y la evaluación de riesgos en casos de brotes son esenciales para un control adecuado de la salud pública. El registro sistemático de equipos facilita la identificación de las fuentes de los brotes y ayuda a acortar su duración. (Gea-Izquierdo, E., 2023)

2.3 BASES LEGALES

La presente investigación se desarrolla conforme a las disposiciones legales nacionales e internacionales vinculadas con la protección de los derechos de propiedad intelectual y los principios éticos que orientan la producción científica en el campo de la salud.

2.4.1. CONSTITUCIÓN POLÍTICA DE LA REPÚBLICA DE PANAMÁ

La Constitución de la República de Panamá (1972), establece que:

TÍTULO III

DERECHOS Y DEBERES INDIVIDUALES Y SOCIALES

CAPÍTULO 1.

GARANTÍAS FUNDAMENTALES

ARTÍCULO 53*." Todo autor, artista o inventor goza de la propiedad exclusiva de su obra o invención, durante el tiempo y la forma que establezca la ley".*

2.4.2. Convenios y tratados internacionales

- El convenio de la OMPI, el instrumento de la Organización Mundial de la Propiedad Intelectual (OMPI), se firmó en Estocolmo el 14 de julio de 1967, entró en vigor en 1970 y fue enmendado en 1979.
- Tratado de la OMPI sobre Derechos de Autor (WCT), adoptado en Ginebra el 20 de diciembre de 1996.

2.4.3. Leyes y decretos nacionales

- Ley 64 del 10 de octubre de 2012 sobre Derechos de Autor y Derechos Conexos.
- Ley 43 de 21 de julio de 2004, que reorganiza el Ministerio de Salud.
- Ley 81 de 26 de marzo de 2019, sobre Protección de Datos Personales.

CAPÍTULO 3 METODOLOGÍA

3.1 Diseño y tipo de estudio

El presente trabajo corresponde a un estudio descriptivo y retrospectivo, basado en una revisión bibliográfica de diseño no experimental. Se realizó una revisión sistemática de documentos, usando el estado del arte como método para guiar el análisis crítico de la información recogida de estudios anteriores sobre el tema.

3.2 Población

La población de este estudio estuvo conformada por un total de siete (7) artículos de investigación seleccionados por su pertinencia temática, centrados en la evaluación ambiental de la presencia de Legionella pneumophila. Estas

investigaciones se seleccionaron a nivel internacional y se enmarcan en el periodo comprendido entre los años 2018 y 2025.

Inicialmente, se revisaron aproximadamente treinta (30) documentos, entre los cuales se incluyeron artículos científicos, revisiones de literatura y otros textos académicos. No obstante, solo se consideraron como parte de la población final aquellos estudios que cumplían con el enfoque investigativo requerido y que respondían de manera directa al objetivo del presente trabajo. Los documentos excluidos, pese a no integrar la muestra final, aportaron elementos relevantes para la construcción del marco teórico y la contextualización del estado del arte. La selección final se realizó a partir de una evaluación crítica de la pertinencia, enfoque metodológico y contribución al análisis del problema de estudio bajo los lineamientos del método PRISMA 2020.

Los estudios analizados que hacen parte de la compilación de evidencias son los siguientes:

Estudio n.º 1: Stagnation arising through intermittent usage is associated with increased viable but nonculturable *Legionella* and amoeba hosts in a hospital water system.

Estudio n.º 2: Environmental Monitoring of *Legionella* in Hospitals in the Campania Region: A 5-Year Study.

Estudio n.º 3: Detection of Legionella pneumophila in the Water Samples of Food Industries and Hospitals in Bangladesh.

Estudio n.º 4: A Study on The Presence of Legionella pneumophila in Hospital Water Samples from Eastern Turkey.

Estudio n.º 5: Evaluation of Legionella pneumophila Decrease in Hot Water Network of Four Hospital Buildings after Installation of Electron Time Flow Taps.

Estudio n.º 6: Water Safety Plan, Monochloramine Disinfection and Extensive Environmental Sampling Effectively Control Legionella and Other Waterborne Pathogens in Nosocomial Settings: The Ten-Year Experience of an Italian Hospital.

Estudio n.º 7: Environmental surveillance of *Legionella pneumophila* in distal water supplies of a hospital for early identification & prevention of hospital-acquired legionellosis.

3.3 Muestra

La muestra corresponde al conjunto de siete (7) documentos y fuentes bibliográficas que cumplieron con los objetivos planteados en la investigación, así como con los criterios de inclusión y exclusión previamente establecidos. Para la selección de la

muestra se consideraron aspectos como la relevancia del contenido, la relación directa con el tema investigado y la actualidad de la información. De esta manera, se logró obtener información confiable y pertinente que contribuye al desarrollo y sustento teórico de la investigación.

Los estudios relacionados que hacen parte de la compilación de evidencia son los siguientes:

Estudio n.º 1: Stagnation arising through intermittent usage is associated with increased viable but nonculturable *Legionella* and amoeba hosts in a hospital water system.

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Estudio n.º 4: A Study on The Presence of Legionella pneumophila in Hospital Water Samples from Eastern Turkey.

Estudio n.º 5: Evaluation of Legionella pneumophila Decrease in Hot Water Network of Four Hospital Buildings after Installation of Electron Time Flow Taps.

Estudio n.º 6: Water Safety Plan, Monochloramine Disinfection and Extensive Environmental Sampling Effectively Control Legionella and Other Waterborne Pathogens in Nosocomial Settings: The Ten-Year Experience of an Italian Hospital.

Estudio n.º 7: Environmental surveillance of *Legionella pneumophila* in distal water supplies of a hospital for early identification & prevention of hospital-acquired legionellosis.

3.4 Procedimiento de recuperación de la información y fuentes documentales

El trabajo es una revisión sistemática de tipo no experimental, de carácter analítico y descriptivo. Esta se realizó bajo los lineamientos del Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020). El estudio se desarrolló durante el periodo comprendido entre mayo y junio de 2026, con el propósito de identificar, evaluar y sintetizar la evidencia científica existente sobre la presencia de Legionella pneumophila en sistemas de aire acondicionado.

Fuentes de información y bases de datos: La búsqueda se realizó en cuatro bases de datos electrónicas principales con cobertura entre 2018 y 2025:

- PubMed/MEDLINE, Scopus (Elsevier)

- ScienceDirect (Elsevier)

Términos MeSH: Estrategia de búsqueda. Se utilizaron descriptores normalizados según los términos MeSH (Medical Subject Headings) y sus equivalentes en español (DeCS). Las combinaciones de búsqueda se formularon mediante operadores booleanos AND y OR. Ecuación de búsqueda general (en inglés): “Visual fatigue” OR “Eye Strain” AND “Ergonomics”

Criterios de inclusión

- Artículos científicos, documentos académicos, informes técnicos que aborden la presencia de Legionella pneumophila en sistemas de aire acondicionado.
- Estudios publicados en revistas indexadas.
- Literatura que se encuentre dentro del rango temporal de 10 años.
- Literatura que aporte información relevante para comprender desde una perspectiva ambiental y sanitaria el tema.

Criterios de exclusión

- Documentos que no estén relacionados directamente con Legionella pneumophila en sistemas de aire acondicionado.
- Estudios que estén fuera del rango temporal establecido.
- Artículos que no sean en idioma español o inglés.

El diagrama de flujo donde queda reflejado el proceso se muestra en la Figura 1.

CAPÍTULO 4: PRESENTACIÓN DE RESULTADOS

4.1 Resultado de análisis

Tabla 1. Análisis individual de los estudios.

N°	Articulo	Tipo de muestra	Método de detección	Factores que favorecen el desarrollo	Principales hallazgos
1	Stagnation arising through intermittent usage is associated with increased viable but non-culturable Legionella andamoeba hosts in a hospital water system	Muestra de agua de la primera descarga del lavabo o la ducha (120) y biopelícula visible del interior del grifo o cabezal de ducha (46)	Se realizó la detección utilizando ensayos de reacción en cadena de la polimerasa cuantitativa (qPCR). Adicionalmente, también se realizó por método de cultivo utilizando agar Legionella suplementado con GVPC (glicina, vancomicina, polimixina B y cicloheximida) y suplemento de crecimiento de Legionella (α-cetoglutarato, tampón/hidróxido de potasio, pirofosfato férrico y L-cisteína). Otro método fue el kit de prueba de	Las temperaturas medidas en el suministro de agua fría, por semana y por mes, dieron un promedio de 21,90 ± 1,84 °C; en el suministro de agua caliente dieron un promedio de 23,67 ± 3,12 °C. por la poca variabilidad de las temperaturas del agua, no se observaron relaciones entre esta y la concentración microbiana.	En este estudio, se identificó que el 31,3 % (n = 21/67) del agua de lavabo, el 18,9 % (n = 10/53) del agua de ducha y el 10,9 % (n = 5/46) de las muestras de biopelícula fueron positivas para el marcador genético de Legionella spp. o L. pneumophila.

			aglutinación de látex para Legionella.		
2	Environmental Monitoring of *Legionella* in Hospitals in the Campania Region: A 5-Year Study	El total de 3365 muestras de agua, 2065 se originaron en grifos y duchas manuales (1485 de agua caliente y 580 de agua fría), 780 de fondos de tanques (en particular, 520 de agua caliente y 260 de agua fría) y 520 de unidades de tratamiento de aire (ATU).	Se inocularon en placas de Petri que contenían medio Legionella Agar Base suplementado con Legionella Growth Supplement (BCYE) y Legionella Selective Supplement (GVPC). Posteriormente, laspresuntas colonias de Legionella se cultivaron tanto en agar BCYE como en agar BCYE sin L-cisteína (agar BCYE-cys). Y la determinación de especies y serogrupos se realizó mediante la prueba de aglutinación de látex y el suero monovalente anti-Legionella pneumophila.	En las muestras tomadas en UTA no se realizaron las medidas de temperatura y cloro residual. Mientras que el resto, se dividieron 2845 muestras entre agua fría y caliente que dieron una temperatura media de 37,5 ◦C (rango de 11,0–91,0 ◦C) para todas las muestras recolectadas. Para el rango de temperatura de 41,0–45,9 ◦C y superior, se observó una disminución en el valor de la media geométrica a medida que	Se confirmó que la especie más frecuente fue L. pneumophila. (con alta detección de los serogrupos 2 a 14). En cuanto al total de serogrupos, el más representado fue el serogrupo 1, Estos resultados sugieren la necesidad de una vigilancia ambiental continua de Legionella y el diagnóstico clínico de serogrupos distintos del serogrupo 1, ya que la prueba más utilizada en muestras humanas muestra una alta sensibilidad solo para este serogrupo. La correlación negativa entre el cloro residual y la presencia de Legionella confirmó

				aumentaba la temperatura. A temperaturas superiores a 62 °C, no se detectó positividad para Legionella.	que la desinfección con cloro es un método eficaz para prevenir la contaminación por Legionella.
3	Detection of Legionella pneumophila in the Water Samples of Food Industries and Hospitals in Bangladesh	Se recolectaron un total de 114 muestras de agua.	El método de detección por cultivo utilizado fue agar de Extracto de Levadura de Carbón Amortiguado (BCYE). Otro método fue la prueba de aglutinación de látex utilizando el Kit de Prueba de Látex Legionella y también se utilizó PCR específica de Legionella pneumophila.	No se realizaron mediciones de factores como temperatura o pH.	En el presente estudio, se detectó un 43 % de L. pneumophila en muestras de agua de torre de enfriamiento de un hospital y dos industrias alimentarias, lo cual está en línea con los datos globales que indican que los sistemas de torre de enfriamiento son el principal reservorio de Legionella. De las 114 muestras que fueron inoculadas en agar (BCYE), solo 30 de ellas se aislaron por tener características típicas de Legionella. El método selectivo de agar BCYE se

					consideró el estándar de oro para detectar L. pneumophila. Sin embargo, no se encontró ninguna L. pneumophila utilizando el medio de crecimiento selectivo en el estudio actual. En contraste con el método basado en cultivos, el ensayo PCR, que tiene como objetivo el gen 16S rRNA de L. pneumophila, resultó ser más sensible para detectar este patógeno, ya que se han detectado (7) L. pneumophila utilizando el ensayo PCR, demostrando la alta eficacia del método. En el ensayo de aglutinación en látex, ninguno de los aislamientos presuntivos de

					Legionella mostró aglutinación positiva.
4	A Study on The Presence of Legionella pneumophila in Hospital Water Samples from Eastern Turkey	Se examinaron un total de 1008 muestras de agua tomadas de varios sistemas de agua de hospitales.	Se utilizaron medios de agar de extracción de carbón tamponado (BCYE) y glicina, vancomicina, polimixina, cicloheximida (GVPC) para el aislamiento de Legionella. Y también se utilizó el método de aglutinación de látex.	Se observó una tasa de positividad más alta en los meses de primavera (33.8 %), seguida de la temporada de verano y otoño; esto se debe a las temperaturas que se registran en estas épocas del año.	Como resultado del estudio, los resultados de la prueba de aglutinación con látex y el serogrupo (SG) de las colonias de Legionella: el 7.04 % (71) de las muestras fueron positivas y el 16.9 % (12) de las muestras positivas eran L. pneumophila SG 1; el 77.46 % (55) de ellas eran L. pneumophila SG 2-14, mientras que el 5.63 % (4) eran no pneumophila.
5	Evaluation of Legionella pneumophila: Decrease in Hot Water Network of Four Hospital Buildings after Installation of Electron Time Flow Taps	Las muestras se tomaron del sistema de agua caliente al que se instalaron en diferentes puntos grifos de flujo temporal (TFT) que se programaron para obtener un flujo de	Se utilizó primero el método de cultivo selectivo agar Legionella BMPA;luego las colonias sospechosas de Legionella se sembraron usando medio BCYE y medio BCYE sin cisteína, Las colonias confirmadas	Antes de la instalación del TFT, la temperatura media del agua caliente en los cuatro hospitales osciló entre 34.35 y 45.63 °C. Tras la implementación del sistema, la temperatura media	La instalación de TFT, junto con la cloración continua y la prefiltración del agua, redujo progresivamente la presencia de *Legionella pneumophila* en los hospitales evaluados, logrando su

		de 192 L/día de cada TFT.	se sometieron a análisis de identificación de especie y serogrupo usando una prueba de aglutinación en látex multipropósito.	aumentó, registrándose valores entre 45.83 y 51.35 °C. Asimismo, la cloración continúa mantenida después de la instalación presentó concentraciones medias de cloro residual entre 0.25 y 0.36 mg/L, evidenciando una mejora en las condiciones de control de la calidad microbiológica del agua.	eliminación completa después de dos meses. Además, el aumento de la temperatura del agua caliente hasta valores cercanos o superiores a 50 °C y la disminución del estancamiento favorecieron el control de la bacteria. Estos resultados indican que la combinación de medidas físicas y químicas de desinfección es eficaz para prevenir la colonización de *Legionella pneumophila* en sistemas de agua hospitalarios.
6	Water Safety Plan, Monochloramine Disinfection and Extensive Environmental Sampling Effectively Control Legionella and Other Waterborne	Se han recogido regularmente un promedio de 250 muestras/año (una ronda de muestreo cada 30–60 días). Estas muestras se recogieron de	Como método de cultivo, se sembró directamente en Agar de Extracto de Levadura de Carbón Amortiguado (BCYE) y en algunos casos, se realizó un subcultivo	No se realizaron mediciones de factores como temperatura o pH.	La evaluación de riesgos determinó que el control térmico y el dióxido de cloro residual no eran suficientes para prevenir la proliferación de

	Pathogens in Nosocomial Settings: The Ten-Year Experience of an Italian Hospital	tanques de almacenamiento, línea principal de agua caliente, circuito de recirculación.	para obtener colonias más puras. Finalmente, de 1 a 3 colonias se examinaron directamente mediante la técnica MALDI-ToF MS usando un VITEK® MS v2.0. Además, cuando se identificó Legionella pneumophila, se determinaron los serogrupos con una prueba de aglutinación.		*Legionella* en los sistemas de agua caliente hospitalarios. Por ello, se implementó una estrategia de desinfección continúa basada en monocloramina, manteniendo concentraciones cercanas a 2.5 mg/L. Los resultados mostraron que esta medida, junto con tratamientos de choque con dióxido de cloro y monocloramina, fue eficaz para restaurar condiciones microbiológicas seguras, eliminando completamente la presencia de Legionella.
7	Environmental surveillance of *Legionella pneumophila* in distal water supplies of a	Se tomaron muestras de treinta grifos en diversas áreas de atención al paciente del	Como método de cultivo se utilizó extracto de levadura y carbón activado tamponado (BCYE)	No se realizaron mediciones de factores como temperatura o pH.	De las 30 muestras analizadas, solo dos hisopados (6,66 %) fueron positivos para *Legionella*

	hospital for early identification & prevention of hospital-acquired legionellosis	hospital. De cada grifo, se recogieron muestras de agua y frotis de la parte interna de la tubería, lo que sumó un total de 60 muestras. Los frotis se recogieron antes que las muestras de agua de la misma fuente.	con y sin antibióticos (polimixina B, cicloheximida, vancomicina). La identificación se confirmó posteriormente mediante aglutinación de látex contra los antisueros 1 y 2-15 de L. pneumophila con un kit comercial. Los aislados que se sospechaba que eran Legionella pero que no aglutinaron con ninguno de los antisueros se sometieron a identificación mediante espectrometría de masas de tiempo de vuelo con ionización/desorción láser asociada a matriz (MALDI-TOF).		*pneumophila* serotipo 2-15, mientras que no se detectó *L. pneumophila* serotipo 1. Los hisopados demostraron una mayor capacidad de detección de microorganismos que las muestras de agua, evidenciando su utilidad para la vigilancia microbiológica de los sistemas hídricos.

Fuente: elaboración propia.

Diagrama de flujo PRISMA (resumen textual)

1. Identificación:
   - Registros encontrados: 30 (PubMed, MEDELINE, Scopus, ScienceDirect)
2. Cribado:
   - Títulos y resúmenes evaluados: 30
   - Registros excluidos: 20
3. Elegibilidad:
   - Textos completos evaluados: 10
   - Estudios excluidos: 3
   - Motivo de exclusión:
   - Falta de metodología de detección y resultados.
   - Falta de información relevante.
4. Inclusión:
   - Estudios incluidos en la revisión: 7

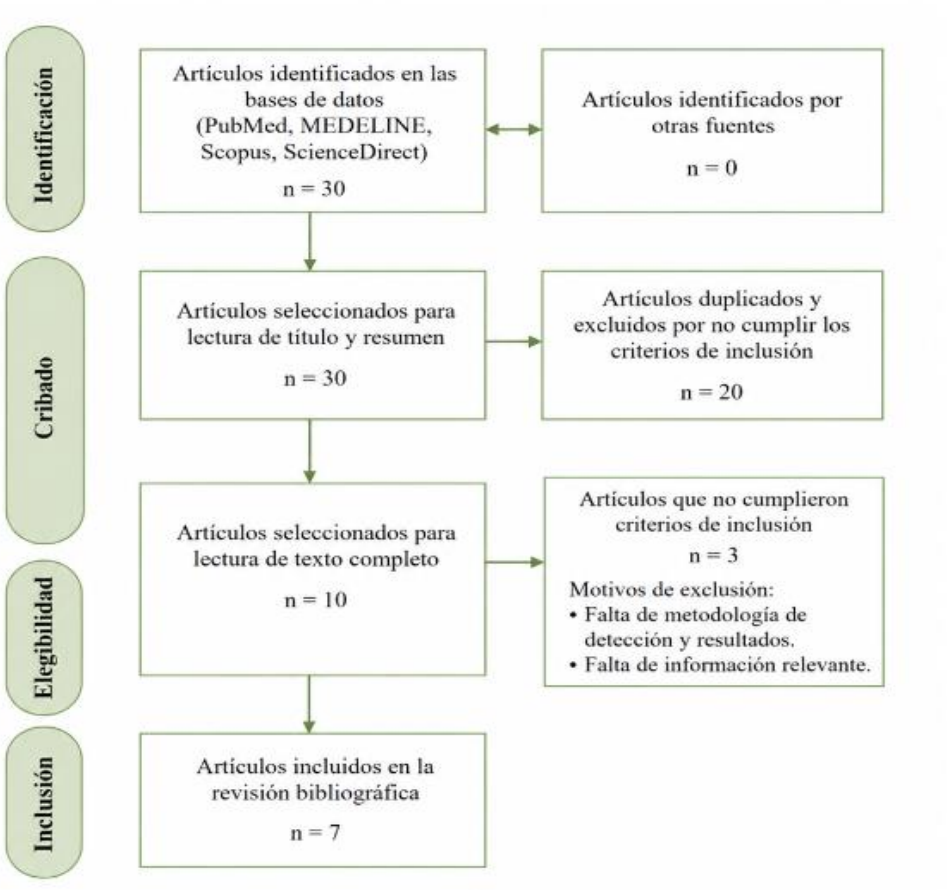

Figura 1. Diagrama de flujo PRISMA, adaptado de la declaración PRISMA 2020.

4.2 Discusión y síntesis de resultados

- Frecuencia de detección de Legionella pneumophila:

  Para entender mejor la tasa de detección de Legionella pneumophila en los 7 artículos revisados, se sintetizaron los datos por continente. Esto ayuda a comprender la situación a nivel mundial.

  En el caso de Asia, los estudios realizados en India y Bangladés muestran una frecuencia relativamente baja de detección de Legionella pneumophila. En India, el muestreo en grifos hospitalarios reveló una positividad del 6.6 %, con predominio del serogrupo 2-15. En Bangladés, aunque los cultivos fueron negativos, la PCR confirmó la presencia de la bacteria en 7 de 114 muestras, lo que evidencia que los métodos moleculares son más sensibles que los convencionales. Estos hallazgos sugieren que la colonización existe, pero puede pasar desapercibida si se depende únicamente de cultivos.

  En Europa, específicamente Italia, la situación es más heterogénea. Los estudios realizados en la región de Campania reportaron un 21 % de positividad, con predominio de serogrupos 2-14 (70.9 %). Sin embargo, en el hospital Papa Giovanni XXIII (Bérgamo), la implementación de un Plan de Seguridad del Agua y desinfección con monocloramina mantuvo la positividad por debajo del 1 % durante diez años, sin casos clínicos registrados. En Pisa, antes de instalar grifos de flujo temporal, los recuentos alcanzaban hasta 1.4×10^5 CFU/L, pero tras la intervención la colonización desapareció en apenas 15 días. Estos resultados muestran que, aunque la colonización puede ser elevada en hospitales europeos, las medidas de control sostenidas logran reducirla a niveles prácticamente nulos.

  En Oceanía, un estudio en Australia reveló que el 21.8 % de las muestras fueron positivas por qPCR, pero solo el 2.5 % resultaron cultivables. Esto indica que gran parte de las bacterias se encontraban en estado viable pero no cultivable (VBNC), lo que subraya la importancia de aplicar métodos moleculares avanzados como qPCR o VFC+qPCR para evitar subdiagnósticos.

- Métodos de monitoreo ambiental:
  En términos de monitoreo, los cultivos en medios selectivos como Agar de Extracto de Levadura y Carbón Tamponado (BCYE) y las pruebas de aglutinación de látex siguen siendo la base, pero se reconoce que su sensibilidad es limitada. Por eso, se han incorporado técnicas como MALDI-TOF MS para identificación precisa y PCR/qPCR para detectar ADN bacteriano, incluso en estados VBNC. Asimismo, se ha demostrado que el muestreo de biofilms mediante hisopos es más eficaz que el agua directa, aumentando el rendimiento de aislamiento. El resultado de un

monitoreo continuo ha evidenciado ser esencial para mantener la colonización bajo control.

- Factores de riesgo:
  Los factores más relevantes incluyeron el estancamiento del agua y el bajo flujo de esta, favoreciendo la formación de biofilms. Las temperaturas entre 25 y 45 °C son óptimas para el crecimiento bacteriano. Además, las tuberías viejas y ramas muertas en hospitales antiguos y la presencia de protozoos como amebas de vida libre actúan como reservorios naturales. Finalmente, los bajos niveles de cloro residual están asociados a mayor colonización. Los tanques de agua caliente se identificaron como puntos críticos de proliferación.

- Medidas de control:
  Las medidas más efectivas registradas para el control de la legionela fueron la desinfección continua con dióxido de cloro y, especialmente, la aplicación de monocloramina in situ, que en el estudio realizado en Italia mantuvo la positividad por debajo del 1 % durante una década. La instalación de grifos de flujo temporal (TFT) eliminó la colonización en apenas dos semanas, demostrando la importancia de evitar el estancamiento. Los Planes de Seguridad del Agua (WSP), recomendados por la OMS, han sido implementados en Italia y Turquía, integrando protocolos de autocontrol y monitoreo periódico, lo que ha permitido reducir significativamente la colonización.

- Impacto en la salud pública.
  El impacto en la salud pública es considerable. En Europa, la letalidad hospitalaria puede alcanzar hasta el 40.5 %, y en brotes nosocomiales se han reportado mortalidades de hasta el 48 %. La carga de enfermedad ha aumentado en los últimos años, con un incremento del 300 % en casos en Europa entre 2011 y 2018. El subdiagnóstico es frecuente, ya que los cultivos convencionales no detectan estados VBNC, ocultando la verdadera magnitud del problema. Sin embargo, la evidencia demuestra que hospitales con planes de seguridad y desinfección avanzada pueden reducir la incidencia clínica a cero, como ocurrió en Italia, confirmando que la prevención y el monitoreo sostenido son claves para mitigar el riesgo y proteger a poblaciones vulnerables.

Conclusiones

La revisión de la literatura permitió identificar qué factores, como la formación de biopelículas, el estancamiento del agua y el mantenimiento inadecuado de sistemas de climatización, contribuyen significativamente a la supervivencia y propagación de este microorganismo bacilo gram negativo.

Por los estudios analizados, se logra identificar factores muy marcados en cuanto a la proliferación de este microorganismo, tales como la falta de mantenimiento, desinfección incorrecta y el estancamiento del agua en zonas "muertas" y en las torres de enfriamiento de las instalaciones, esto acentuándose en las estaciones de primavera, seguido por verano y otoño, que es cuando las temperaturas son más optimas.

Se logró identificar que las medidas más efectivas para el control de *Legionella* en los sistemas de agua hospitalarios son aquellas que combinan estrategias de desinfección continua, control de la infraestructura y monitoreo permanente. Entre las intervenciones con mejores resultados destaca la desinfección con monocloramina in situ, la cual logró mantener niveles de positividad inferiores al 1 %. La instalación de grifos de flujo temporal demostró ser una medida eficaz para eliminar la colonización en un corto plazo al prevenir el estancamiento del agua.

Con el análisis crítico realizado, se llegó a la conclusión de que el sistema de aire acondicionado no es el problema per se; en cambio, es el conjunto que forma el sistema de climatización, empezando desde las torres de enfriamiento, donde, si las tuberías que transportan el agua no tienen el mantenimiento adecuado, están contaminadas. El agua se rocía dentro de la torre mientras un ventilador hace pasar aire a través de ella. Al entrar en contacto, una pequeña porción de agua se evapora, absorbiendo el calor y enfriando el resto del agua para que pueda volver a ser utilizada en el ciclo de climatización, lo que vuelve las salidas del aire acondicionado el final donde se pueden liberar aerosoles.

Recomendaciones

- Implementación de programas de mantenimiento preventivo en los sistemas de aire acondicionado y en las torres de enfriamiento.
- Desarrollar cronogramas y registros de limpieza, desinfección e inspección de los sistemas de climatización y agua para asegurar un buen control microbiológico.
- Implementar un seguimiento periódico de los parámetros fisicoquímicos del agua, tales como la temperatura, el pH y los niveles de desinfectantes, para mitigar la proliferación.
- Concientizar y capacitar al personal de mantenimiento sobre la importancia de este tipo de microorganismos que crean biofilms.
- La implementación de grifos de flujo temporal (TFT) en zonas del hospital en desuso o con poco uso.
- Desarrollar estudios sobre los desinfectantes del agua en uso, ya que algunos pueden dañar los sistemas de tubería, como lo es el dióxido de cloro, dada su incompatibilidad con las tuberías de PP-R; en estos casos se recomendaría la utilización de desinfectantes como la monocloramina.
- Llevar a cabo evaluaciones periódicas de la infraestructura hidráulica y de climatización para identificar zonas de estancamiento o “puntos muertos” que favorezcan la proliferación del microorganismo.
- Implementar programas de vigilancia ambiental periódica en torres de enfriamiento y tanques de almacenamiento de agua, con el fin de detectar tempranamente la presencia de Legionella pneumophila y evaluar la eficacia de las medidas de control implementadas.
- Fomentar la realización de investigaciones locales sobre la presencia de Legionella pneumophila en sistemas de climatización y agua hospitalarios, ya que existe una limitada cantidad de estudios en muchos países de la región.

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Anexos

Anexo 1. Capturas del artículo n°1 titulado: Stagnation arising through intermittent usage is associated with increased viable but non-culturable Legionella and amoeba hosts in a hospital water system.

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Stagnation arising through intermittent usage is associated with increased viable but non culturable *Legionella* and amoeba hosts in a hospital water system

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Hospital water systems are a significant source of *Legionella*, resulting in the potentially fatal Legionnaires' disease. One of the biggest challenges for *Legionella* management within these systems is that under unfavorable conditions *Legionella* transforms itself into a viable but non culturable (VBNC) state that cannot be detected using the standard methods. This study used a novel method (flow cytometry-cell sorting and qPCR [VFC+qPCR] assay) concurrently with the standard detection methods to examine the effect of temporary water stagnation, on *Legionella* spp. and microbial communities present in a hospital water system. Water samples were also analyzed for amoebae using culture and *Vermamoeba vermiformis* and *Acanthamoeba* specific qPCR. The water temperature, number and duration of water flow events for the hand basins and showers sampled was measured using the Enware Smart Flow® monitoring system. qPCR analysis demonstrated that 21.8% samples were positive for *Legionella* spp., 21% for *L. pneumophila*, 40.9% for *V. vermiformis* and 4.2% for *Acanthamoeba*. All samples that were *Legionella* spp. positive using qPCR (22%) were also positive for VBNC *Legionella* spp.; however, only 2.5% of samples were positive for culturable *Legionella* spp. 18.1% of the samples were positive for free-living amoebae (FLA) using culture. All samples positive for *Legionella* spp. were also positive for FLA. Samples with a high heterotrophic plate count (HPC $\geq 5 \times 10^3$ CFU/L) were also significantly associated with high concentrations of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila* ($p < 0.01$) and *V. vermiformis* ($p < 0.05$). Temporary water stagnation arising through intermittent usage (< 2 hours of usage per month) significantly ($p < 0.01$) increased the amount of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila*, and *V. vermiformis;* however, it did not significantly impact the HPC load. In contrast to stagnation, no relationship

was observed between the microbes and water temperature. In conclusion, *Legionella* spp. (DNA and VBNC) was associated with *V. vermiformis*, heterotrophic bacteria, and stagnation occurring through intermittent usage. This is the first study to monitor VBNC *Legionella* spp. within a hospital water system. The high percentage of false negative *Legionella* spp. results provided by the culture method supports the use of either qPCR or VFC+qPCR to monitor *Legionella* spp. contamination within hospital water systems.

KEYWORDS

Legionnaires' disease, water safety plan, building plumbing systems, free-living amoebae, potable water

1 Introduction

Legionella is an opportunistic premise plumbing pathogen and etiological agent of Legionnaires' disease (LD), a potentially fatal pneumonia like infection (Cunha et al., 2016). *Legionella* is ubiquitous in natural and engineered water systems and transmitted through aspiration or inhalation of *Legionella* contaminated water or aerosols (Schwake et al., 2021). Globally the incidence of LD has been increasing. In 2021, the US Centers for Disease Control and Prevention (CDC) reported 8260 confirmed cases of LD in USA (Centers for Disease Control and Prevention, 2022). In Australia, 524 confirmed cases of legionellosis were reported in 2020 (Australian Government Department of Health and Aged Care, 2021). According to the European Centre for Disease Prevention and Control (ECDC) 11,298 confirmed cases of LD were documented across European countries in 2019. However, in 2020 the number decreased to 8,372; this reduction may be associated with COVID-19 pandemic lockdown restrictions or a decrease in focus on LD. In 2021, 10,723 confirmed cases of LD were documented of which 5.4% were nosocomial infections (The European Legionnaires' Disease Surveillance Network, 2022). The actual number of legionellosis cases is understated, because in the majority of cases Pontiac fever remains unnoticed and the etiological agent of pneumonia remains unrecognized (Cassell et al., 2019). There are at least 60 distinct species of *Legionella*, with *L. pneumophila* sg.1 being the most common cause of outbreaks (Khodr et al., 2016; Miyashita et al., 2020). Initially, cooling towers were considered to be the main source of *Legionella* spp., but subsequent investigations have identified that engineered water systems are a major source of LD (Kanarek et al., 2022). Those at greatest risk of infection are the elderly and immunocompromised individuals, and as such nosocomial outbreaks associated with hospital engineered water systems are of significant concern (Bartram et al., 2007).

A range of factors influence the survival and persistence of *Legionella* spp. in hospital water systems including: biofilms, nutrients, disinfectants, protozoa hosts, water temperature, flow dynamics and stagnation (Abdel-Nour et al., 2013; Whiley et al., 2017; Nisar et al., 2020b). Naturally, *Legionella* spp. infects and survives within a wide range of polyphyletic protozoan hosts, with *Acanthamoeba* and *Vermamoeba* the most commonly identified hosts in potable water (Boamah et al., 2017; Best and Abu Kwaik, 2018; Nisar et al., 2020a). Intracytoplasmic *Legionella* spp. are protected from adverse environmental conditions (Best and Abu Kwaik, 2018), with *Legionella* spp. released from host protozoa more virulent and pathogenic in nature (Fields et al., 2002; Boamah et al., 2017). Additionally, *Legionella* spp. intrinsically tolerate water disinfection treatments by entering into a metabolically inactive but highly resistant and potentially pathogenic "viable but non-culturable" (VBNC) state (Kirschner, 2016). Under suitable environmental conditions, and in the presence of protozoa hosts, VBNC *Legionella* spp. can resuscitate back into a culturable state (Dietersdorfer et al., 2018). VBNC *Legionella* spp. are a significant challenge to water quality management as they cannot be detected using the standard culture-based method (International Organization for Standardization, 2017; Standards Australia, 2017). *Legionella* spp. specific quantitative PCR (qPCR) assay is an alternative method typically used to detect the genomic load of *Legionella* spp. (International Organization for Standardization, 2019); however, it cannot distinguish between culturable, dead and VBNC *Legionella* spp. (Kirschner, 2016). As such, there are currently limited studies that investigate the survival of VBNC *Legionella* spp. in engineered water systems.

Water stagnation in engineered water systems is categorized into two different types; permanent, and temporary stagnation (Peter and Routledge, 2018; Nisar et al., 2020b). Permanent stagnation is complete stagnation of plumbing structures, such as dead-ends and dead-legs (Nisar et al., 2020b). However, in engineered water systems, water can also stagnate in storage tanks, plumbing piping network, and within components at the water outlets for a few hours to weeks and even months (Bartram et al., 2007; Manuel et al., 2009). This type of water stagnation is known as intermittent or temporary stagnation (Manuel et al., 2009; Peter and Routledge, 2018). The relationship between *Legionella* spp. and permanent stagnation is well characterized (Totaro et al., 2018; Nisar et al., 2020b). However, less is known about the relationship between *Legionella* spp. and temporary stagnation. Therefore, this study examined the role of temporary stagnation arising through intermittent water usage on the persistence of *Legionella* spp. and free-living amoebae (FLA) within a hospital water system.

This study was the first study to utilize a novel method to enumerate VBNC *Legionella* spp. and *L. pneumophila* from environmental water samples and investigate relationships with protozoan hosts. This study utilized the Enware Smart® Flow monitoring system to examine the relationships between water flow (arising through water outlet usage) and temperature with *Legionella* spp., *L. pneumophila* and amoeba hosts. The specific aims of this study were as follows, to: (1) determine the prevalence of *Legionella* spp./*L. pneumophila* and FLA in a hospital water system; (2) examine the relationship between *Legionella* spp. and potential protozoan hosts; and (3) monitor the effect of sampling phases (months), water temperature, flow dynamics and stagnation on persistence of *Legionella* spp. in the hospital water system. To our knowledge, this is the first comprehensive study that has quantified VBNC *Legionella* spp. and FLA from a hospital water system under dynamic flow and temperature conditions.

2 Materials and methods

2.1 Sample collection and processing

From March 2021 to June 2022, water (n = 120) and biofilm (n = 46) samples were collected from the engineered water system of an Australian hospital located in New South Wales, Australia. The sampling was done in different phases, where the categorization was: March 2021 as phase 1, April 2021 as phase 2, November 2021 as phase 3 and June 2022 as phase 4. All water and biofilm samples were collected, transported and stored as recommended by standard guidelines (International Organization for Standardization, 2018; Centers for Disease Control and Prevention, 2019). For the water samples, 1 L first flush hand basin or shower water was collected in sterile wide-mouth screw capped plastic bottles (2105-0032, Nalgene™). For the biofilm samples, visible biofilm was scraped from the inside of tap faucet or shower head using sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®), then 5 to 10 mL of water was added and placed with the swab in a sterile screw capped tube. For both the water and biofilm samples, 0.5 mL 0.1 N sodium thiosulfate (124270010, ACROS Organics™) was added to neutralize pre-existing chlorine-based chemical disinfectants. All samples were transported and kept at 5 ± 2°C and processed within 72 hours. The samples were vacuum filtered through 47 mm diameter 0.22 µm polycarbonate membrane (GTTP04700, Isopore™). The filtered residues were resuspended in 3 mL sterile distilled water. This sample suspension was used for further microbiological and molecular testing.

2.2 Water flow and temperature data

Parameters related to water temperature and flow dynamics were monitored in the hospital water system using the Enware Smart® Flow monitoring system. Briefly, this monitoring system measures water system delivery temperatures using temperature probes located at the hot water inlet, cold water inlet, and outlet of the thermostatic mixing valves (TMV) and the hot water inlet and cold water inlet of hand basin faucets. Water flow was measured using flow switches located at the hot water inlet and cold water inlet of both the TMVs and hand basin faucets (Whiley et al., 2019). The temperature data of the hot water supply, cold water supply and outlet was collected for the entire duration of the sampling period. For analysis these measurements were separated into a period one week and one month prior to a water sampling event. In terms of flow regime, the total duration (hours) and number (counts) of flushing events for a period of one week and one month prior to sampling were recorded. The total duration (hours) of flushing events were divided into low and high flow regimes with categorization as: low flow regime; 0 to < 2 hours per month, and high flow regime; ≥ 2 to 40 hours per month.

2.3 Molecular analysis

Quantification of *Legionella* spp. (16 rDNA gene) and *L. pneumophila* (*mip* gene) was performed using ISO/TS12869:2019 quantitative polymerase chain reaction (qPCR) assays (International Organization for Standardization, 2019). The 18S rDNA gene was amplified for the quantification of *Acanthamoeba* and *Vermamoeba vermiformis* (Qvarnstrom et al., 2006; Scheikl et al., 2016). *Legionella* spp. (GenBank Acc CP021281), *L. pneumophila* (GenBank Acc KR902705), *Acanthamoeba castellanii* (GenBank Acc U07413) and *V. vermiformis* (GenBank Acc KT185625) gBlocks gene fragments (IDT™) were used as a positive control and for the preparation of a standard curve using ten-fold serial dilutions. Using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.), genomic DNA was extracted from each water and biofilm sample before being subjected to a qPCR assay. The qPCR reaction mixture consisted of microbe-specific primers (Bio-Rad Laboratories Ltd.), 1X PCR reaction buffer (2X SsoAdvanced™ universal probes supermix:172-5281, Bio-Rad Laboratories Ltd.), and DNA template. To detect the potential presence of environmental inhibitors of the qPCR assays, both the purified and a one in ten dilution of extracted DNA was used as template (Hayes-Phillips et al., 2019; Nisar et al., 2022). Using a Rotor-Gene Q thermal cycler (Qiagen Ltd.), each template DNA was subjected to the qPCR assay in triplicate (Nisar et al., 2022). All fluorescence labelled probes and primers used in this study are presented in Table S1 (Supplementary Material).

2.4 Microbiological analysis

Isolation of culturable *Legionella* spp. and *L. pneumophila* was performed in accordance with the standard guidelines (International Organization for Standardization, 2017; Standards Australia, 2017). Briefly, samples were heat treated (50 ± 1°C for 30 ± 2 minutes) and/or acid treated (HCl-KCl buffer treatment for 5 ± 0.5 minutes) to reduce the contamination of interfering microbes. An aliquot of treated sample was then spread on *Legionella* agar (CM1203, Oxoid Ltd.) supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (α-ketoglutarate,

buffer/potassium hydroxide, ferric pyrophosphate, and L-cysteine: SR0110C, Oxoid Ltd.). The inoculated plates were incubated at 37 ± 1°C for 7 days and examined every day. Suspected *Legionella*-like colonies were counted from each plate and evaluated by *Legionella* latex agglutination test kit (DR0800, Oxoid Ltd.). This kit identifies genus *Legionella* and further characterizes various species and serogroups with overall 99% sensitivity and 100% specificity. Furthermore, all *Legionella*-like colonies were confirmed through *Legionella* spp. specific qPCR assays. To determine the heterotrophic plate counts (HPC), an aliquot from each sample was inoculated on R_2A agar (CM0906, Oxoid Ltd.) and incubated at 35 ± 1°C. The colonies were counted after 2, 5 and 7 days of incubation. The results for *Legionella* spp. and heterotrophic bacteria were expressed in colony forming units (CFU)/L for water samples and CFU for the biofilm samples. Isolation of culturable FLA was performed by inoculating an aliquot of each sample on heat-inactivated (57°C for 45 minutes) *Escherichia coli* American Type Culture Collection 700891™ supplemented 1.5% non-nutrient agar (Eco-NNA: CM0003, Oxoid Ltd.) (Nisar et al., 2022). The plates were incubated at 25 ± 1°C for 14 days and amoebal growth was examined daily using an inverted light microscope (AMEFC4300, EVOS™ FL, Thermo Fisher Scientific). All monoxenic amoebae cultures were characterized by microscopic examination and sequence analysis of 18S rDNA gene.

2.5 Quantification of VBNC *Legionella* spp. and *L. pneumophila*

VBNC *Legionella* spp. and *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR (VFC+qPCR) assay (Nisar et al., 2023). Briefly, 300 µL sample suspension was resuspended in 200 µL of filter sterilized staining buffer (0.01% Tween-20 and 1 mM EDTA in 1X PBS, pH 7.4 ± 0.1), followed by addition of 48 µM propidium iodide (PI) and 420 nM thiazole orange (TO) dyes (cell viability kit Cat # 349480, Becton Dickinson, Franklin Lakes, USA). The mixture was incubated at 5°C for 15 minutes. Then, using a FACS Aria Fusion instrument, (Becton Dickinson, Franklin Lakes, USA) cells were analyzed and segregated into dead (PI/TO), alive (potentially culturable: TO), and injured (potentially VBNC: PI/TO) cell populations. From each sample, the injured cell fraction was isolated and subjected to DNA extraction and quantification of *Legionella* spp. and *L. pneumophila* gene markers (Nisar et al., 2023).

2.6 Data analysis

The data are described in logarithmic form with base 10 ($\log_{10}$). The percentage of both bacterial and amoebae isolates was determined based on phases or contamination levels and plotted in Microsoft® Excel®. Statistical calculations were made using R studio (version 4.2.2) and graphically presented by using "ggplot2 (version 3.3.6)" package (Wickham, 2016). Briefly, the Shapiro-Wilk test was used to assess normality of analyzed quantitative parameters. For comparison of the means of the quantitative parameters (i.e., either GU or CFU of *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*), a non-parametric Kruskal-Wallis test was used. Finally, the non-parametric Spearman's correlation (ρ) test was used to evaluate relationships among different variables (i.e., *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*). A statistically significant difference among the quantitative parameters was defined by p values of less than 0.05.

3 Results

3.1 Occurrence of *Legionella* spp. and *L. pneumophila*

Table 1 presents an overview of the percentage of samples identified as positive for *Legionella* spp. and FLA using the different detection methods. All samples that were qPCR positive for *Legionella* spp. were also positive for FLA and VBNC *Legionella*. Specifically, 21.7% (n = 36/166) of total samples were positive for *Legionella* spp. DNA (16S rDNA gene) with a concentration range of 9×10^2 to 1.5×10^6 GU (Tables 1, 2). *L. pneumophila* DNA (*mip* gene) was present in 21% samples (n = 35/166) with a concentration ranging from 3.5×10^2 to 9×10^4 GU (Tables 1, 2). All *L. pneumophila* positive samples were also positive for *Legionella* spp. During phase 1, 58.06% (n = 18/31) of the samples tested positive for *Legionella* spp. DNA, whereas in the 2nd phase 7.31% (n = 3/41), and 4th phase 28.84% (n = 15/52) of the collected samples tested positive for *Legionella* spp. DNA (Figure 1). However, in phase 3 none of the samples were positive for either *Legionella* spp. or *L. pneumophila* DNA. Standard culturing demonstrated that only four samples (two in phase 1 and two in phase 4) were positive for culturable *Legionella* spp., which were identified as non-*pneumophila Legionella* using serology and qPCR. The VFC+qPCR assay demonstrated that all samples positive for either *Legionella* spp. or *L. pneumophila* DNA (according to the qPCR assay) also contained VBNC cells (Figure 1; Table 1). Therefore, of the 36 samples that were positive for VBNC *Legionella* spp., the standard microbiological culturing assay returned a false negative result for 32 of them (88.9%). For analysis, the VBNC *Legionella* spp. and *L. pneumophila* samples were categorized into three groups based on concentration i.e., low ($< 10^3$ GU/L), medium ($\geq 10^3$ to 10^4 GU/L) and high ($> 10^4$ GU/L) contamination. Based on this grouping it was found that in phase 1, 14.3% (n = 4/31) of the water samples were positive for high VBNC *Legionella* spp. contamination, whereas in the 4th phase 33.3% (n = 10/52) water samples were positive for high VBNC *Legionella* spp. contamination (Supplementary Material, Figure S1A). It was found that the lower level of VBNC *L. pneumophila* occurred more frequently in the samples collected during phase 1 (53.5%, n = 15/31) and 4 (23.3%, n = 7/52). Only 6.7% (phase 4: n = 2/52) water samples contained high levels of VBNC *L. pneumophila* contamination (Supplementary Material, Figure S1B). Based on sampling sites it was found that in hand basin water, 13.4% (n = 9/

TABLE 1 Prevalence of *Legionella* spp., *Vermamoeba vermiformis*, *Acanthamoeba* and total free-living amoeba in a hospital water system using different detection methods.

Sample (n)	*Number of Legionella* positive samples (%)					Number of free-living amoeba positive samples (%)		
	qPCR assay		VFC+qPCR		Culture assay	qPCR assay		Culture assay
	Legionella	*L. pneumophila*	*Legionella*	*L. pneumophila*		*Acanthamoeba*	*V. vermiformis*	
Sampling phase 1 (March 2021)								
Hand basin water (n = 16)	11	11	11	11	1	3	15	9
Shower water (n = 12)	4	4	4	4	1	0	9	6
Tap faucet biofilm (n = 3)	3	3	3	3	0	1	2	1
Total (n = 31)	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**2 (6.45%)**	**4 (12.9%)**	**26 (83.87%)**	**16 (51.61%)**
Sampling phase 2 (April 2021)								
Hand basin water (n = 17)	2	2	2	2	0	0	11	8
Shower water (n = 13)	1	1	1	1	0	0	4	2
Tap faucet biofilm (n = 11)	0	0	0	0	0	0	5	0
Total (n = 41)	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**0**	**0**	**20 (48.78%)**	**10 (24.39%)**
Sampling phase 3 (November 2021)								
Hand basin water (n = 18)	0	0	0	0	0	1	0	1
Shower water (n = 14)	0	0	0	0	0	0	0	0
Tap faucet biofilm (n = 10)	0	0	0	0	0	0	1	1
Total (n = 42)	**0**	**0**	**0**	**0**	**0**	**1 (2.38%)**	**1 (2.38%)**	**2 (4.76%)**
Sampling phase 4 (June 2022)								
Hand basin water (n = 16)	8	7	8	7	0	1	10	0
Shower water (n = 14)	5	5	5	5	0	0	7	0
Tap faucet biofilm (n = 22)	2	2	2	2	2	1	4	2
Total (n = 52)	**15 (28.84%)**	**14 (26.92%)**	**15 (28.84%)**	**14 (26.92%)**	**2 (3.84%)**	**2 (3.84%)**	**21 (48.38%)**	**2 (3.84%)**

67) samples were positive for high VBNC *Legionella* spp. contamination, whereas in shower water, 9.4% (n = 5/53) samples were positive for high VBNC *Legionella* spp. contamination (Supplementary Material, Figure S2A). However, the majority of hand basin (22.4%, n = 15/67) and shower (15.1%, n = 8/53) water samples contained low levels of VBNC *L. pneumophila* contamination (Supplementary Material, Figure S2B). Overall, both qPCR and VFC+qPCR assays clearly demonstrated that the standard culturing assay is frequently unable to detect *Legionella* spp./*L. pneumophila* present in the hospital water system.

3.2 Occurrence of heterotrophic bacteria and FLA

Both the hand basin and shower water samples contained HPC counts ranging from 10 to 1.5×10^5 CFU/L (Table 2). In the biofilm samples the HPC load ranged from 15 to 7.5×10^4 CFU/sample (Table 2). In case of FLA, the *V. vermiformis* gene marker was present in 40.9% (n = 68/166) of samples with concentrations ranging from 7.5×10^2 to 7.5×10^7 GU (Tables 1, 2). The *Acanthamoeba* gene marker was detected only in hand basin

TABLE 2 The minimum and maximum microbial concentrations present in the positive water and biofilm samples.

Microbes	Minimum concentration	Maximum concentration
Hand basin water (n = 67)		
Legionella DNA (GU/L)	1×10^3	1.5×10^6
VBNC *Legionella* (GU/L)	2.5×10^2	6.5×10^5
L. pneumophila DNA (GU/L)	3.5×10^2	9×10^4
VBNC *L. pneumophila* (GU/L)	6×10^2	8.5×10^4
Vermamoeba vermiformis (GU/L)	7.5×10^2	7.5×10^7
Acanthamoeba (GU/L)	1×10^3	5×10^3
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Shower water (n = 53)		
Legionella DNA (GU/L)	9×10^2	7×10^4
VBNC *Legionella* (GU/L)	3.5×10^2	2.5×10^4
L. pneumophila DNA (GU/L)	3.5×10^2	9.5×10^3
VBNC *L. pneumophila* (GU/L)	70	4.5×10^3
Vermamoeba vermiformis (GU/L)	1×10^3	4×10^7
Acanthamoeba (GU/L)	0	0
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Tap faucet biofilm (n = 46)		
Legionella DNA (GU)	1×10^4	3.5×10^5
VBNC *Legionella* (GU)	1.5×10^2	3×10^4
L. pneumophila DNA (GU)	7.5×10^2	1.5×10^4
VBNC *L. pneumophila* (GU)	1×10^2	1×10^4
Vermamoeba vermiformis (GU)	1×10^3	1×10^6
Acanthamoeba (GU)	4.5×10^3	8×10^3
Heterotrophic plate count (CFU)	15	7.5×10^4

water (3.01%, n = 5/166) and biofilm samples (1.2%, n = 2/166) with a range of 1×10^3 to 8×10^3 GU (Tables 1, 2). Culturable amoebae were identified in 18.1% (n = 30/166) samples; however, due to fungal overgrowth 13 isolates were unable to develop monoxenic cultures, of these 13, five isolates showed acrasid amoebae-like morphology. Only 17 isolates developed monoxenic cultures which were further characterized on the basis of cellular morphology and sequence analysis of 18S rDNA gene. Light microscopy revealed that isolates harbored monotactic morphotype and developed spherical cysts consisting of distinct inner and outer walls. Based on 18S rDNA sequencing, all these monoxenic isolates were identified as *V. vermiformis*.

3.3 Relationship among *Legionella* spp., HPC, and FLA

All shower and hand basin water samples were classified into two groups based on the HPC levels i.e., low (10 to $< 5 \times 10^3$ CFU/L) and high ($\geq 5 \times 10^3$ CFU/L) contamination. Kruskal-Wallis analysis demonstrated that quantity of both *Legionella* spp. DNA and VBNC *Legionella* spp. were significantly ($p < 0.001$) higher in water samples with high levels of HPC load (Table 3; Figure 2). Similarly, water samples having greater levels of HPC load harbored significantly higher concentrations of *L. pneumophila* DNA (Kruskal-Wallis test, $p < 0.01$), VBNC *L. pneumophila* (Kruskal-Wallis test, $p < 0.01$), and *V. vermiformis* (Kruskal-Wallis test, $p < 0.05$) (Tables 3; Figure 2). Furthermore, all samples characterized as positive for *Legionella* spp./*L. pneumophila* (DNA, culturable, and VBNC cells) were also positive for either the *V. vermiformis* gene marker or culturable amoebae. Furthermore, Spearman's analysis demonstrated both *Legionella* spp./*L. pneumophila* DNA and *Legionella* spp./*L. pneumophila* VBNC cells were positively correlated ($p < 0.001$) with *V. vermiformis* (Table 4). Overall, these results suggested that in hospital water system, high levels of HPC load and *V. vermiformis* are positively associated with both *Legionella* spp./*L. pneumophila* DNA and VBNC cells.

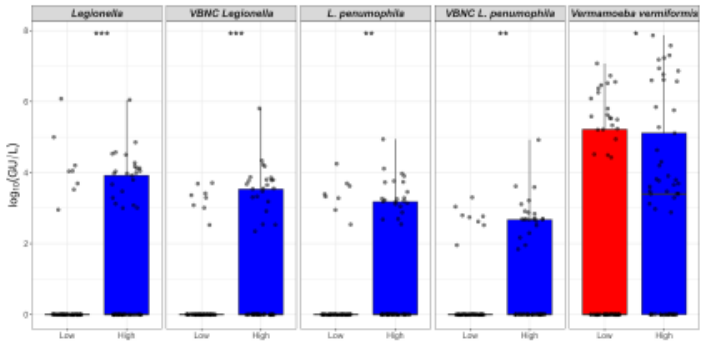

FIGURE 1
Prevalence (%) of *Legionella* spp. and free-living amoeba in a hospital water system. A total of 166 water (hand basin and shower) and biofilm (tap faucet) samples were collected in March 2021 (Phase 1), April 2021 (Phase 2), November 2021 (Phase 3), and June 2022 (Phase 4). Total amounts of *Legionella* spp./*L. pneumophila*, *Acanthamoeba*, and *Vermamoeba vermiformis* were detected and quantified by qPCR assays. Culturable *Legionella* spp. and amoebae were detected by standard microbiological culturing procedures. VBNC *Legionella* spp. and VBNC *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR assay. The p values are: *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

TABLE 3 Effect of flow regime (flushing duration one-month prior to sampling) and heterotrophic plate counts of hand basin and shower water microbes.

Microbes in hand basin and shower water	Flow regime (one-month prior sampling) *		Heterotrophic plate count **	
	Low (0 to < 2 hours/month)	High (≥ 2 to 40 hours/month)	Low (10 to < 5×10^3 CFU/L)	High (≥ 5×10^3 to 1.5×10^5 CFU/L)
Total *Legionella* (GU/L)	1.783 ± 2.043	0.637 ± 1.559	0.532 ± 1.443	1.604 ± 2.015
VBNC *Legionella* (GU/L)	1.560 ± 1.771	0.551 ± 1.366	0.413 ± 1.100	1.455 ± 1.839
Total *L. pneumophila* (GU/L)	1.472 ± 1.671	0.495 ± 1.284	0.429 ± 1.148	1.326 ± 1.698
VBNC *L. pneumophila* (GU/L)	1.206 ± 1.372	0.416 ± 1.107	0.344 ± 0.921	1.106 ± 1.442
***Vermamoeba vermiformis* (GU/L)**	3.578 ± 2.835	1.755 ± 2.508	1.983 ± 2.763	2.952 ± 2.755
Heterotrophic plate count (CFU/L)	3.742 ± 1.010	3.656 ± 0.855	–	–

Data is log transformed and shown as mean ± standard deviation.
*Microbial loads (except heterotrophic plate count) in the low flow regime are significantly higher than in the high flow regimes (Kruskal-Wallis analysis, $p < 0.05$).
**Microbial loads in high heterotrophic plate counts are significantly higher than low heterotrophic plate counts (Kruskal-Wallis analysis, $p < 0.05$).

3.4 Influence of flow regimes and on *Legionella* spp., HPC, and FLA

The total duration (hours) of flushing events for one month prior to sampling, was categorized into: low (0 to < 2 hours/month) and high (≥ 2 to 40 hours/month) flow regimes. The Kruskal-Wallis analysis indicated that the concentrations of *Legionella* spp. DNA ($p < 0.01$), *L. pneumophila* DNA ($p < 0.01$), VBNC *Legionella* spp. ($p < 0.001$), VBNC *L. pneumophila* ($p < 0.001$) and *V. vermiformis* DNA ($p < 0.05$), were all higher in low flow regimes compared with high flow regimes (Table 3; Figure 3). When the total duration (hours) of flushing events for only one week prior to sampling was examined, no association was observed with any of the microbial concentrations measured. The HPC load did not show any measurable difference in the low vs high flow regimes either one month or one week prior to sampling (Table 3). In contrast with the total flow duration, the total number of flow counts (number of flushing events) for either one week or one month prior to sampling was not associated with any significant change in any of the microbial concentrations measured. In conclusion, a month of reduced usage (< 2 hours water flushing per month) supports the proliferation of *Legionella* spp./*L. pneumophila* and *V. vermiformis* in hospital water system.

3.5 Influence of water temperature and on *Legionella* spp., HPC, and FLA

The water outlets (hand basins and showers) of the hospital water system received water from both the cold water supply and hot water supply (Supplementary Material; Figures S3, S5). The temperature data for each sample location were averaged over one week and one month prior to sample collection (Supplementary Material; Figures S3, S5). The average temperatures (mean ± SD)

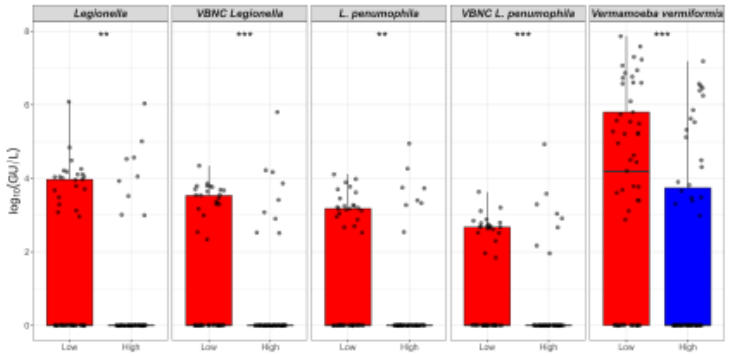

FIGURE 2
Relationship between the heterotrophic plate count and *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents HPC level that is categorised into low (10 to < 5×10^5 CFU/L) and high (≥ 5×10^5 to 1.5×10^5 CFU/L) contamination. Y-axis represents $\log_{10}$(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*. The p values are: $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

measured from the cold water supply were (21.78 ± 1.98°C per week and 22.01 ± 1.69°C per month), hot water supply (23.64 ± 3.15°C per week and 23.69 ± 3.08°C per month) and outlet water (23.74 ± 2.43°C per week and 23.76 ± 1.95°C per month). No relationships between microbial concentration and water temperatures were observed. This is likely due to the average water temperature being similar for both hot and cold water supplies, with increases in hot water temperature occurring through hot water usage having a limited effect on the overall average temperature due to the periods of stagnation and inactivation occurring in between usages (Supplementary Material, Figures S4, S6).

4 Discussion

In this study, it was identified that 31.3% (n = 21/67) hand basin water, 18.9% (n = 10/53) shower water and 10.9% (n = 5/46) biofilm samples were positive for either *Legionella* spp. or *L. pneumophila* gene marker (Table 1). According to the literature, the majority of engineered water systems of hospital and healthcare facilities are contaminated with *Legionella* spp. or *L. pneumophila*. In Poland, 74.7% of water samples from hospitals and other large building structures tested positive for *Legionella* spp., and *L. pneumophila* sg2-14 was the most prevalent serogroup (Sikora et al., 2015). A similar study conducted in Hungary that examined water samples from healthcare facilities and other buildings showed that 60% samples were positive for *Legionella* spp. (predominantly *L. pneumophila* sg2-14) (Barna et al., 2016). A study conducted in 20 different hospitals in Spain reported that 37.2% of water samples were colonized with *L. pneumophila* sg1 and *L. pneumophila* sg2-14 (Sabrià et al., 2004). In Taiwan, 63% of samples collected from hospital water systems tested positive for *Legionella* spp. and *L. pneumophila* sg1 (Yu et al., 2008). Comprehensive national surveillance studies conducted in 13 different states of the USA reported that 70% of hospital water systems were contaminated with *Legionella* spp. (Stout et al., 2007). A recent study conducted in Australia detected 41% samples of water and biofilms from hospital and residential buildings were colonized with *Legionella* spp. (Nisar et al., 2022). The lower *Legionella* spp. prevalence in this study could be due to the fact this was a case study of a single hospital that has been proactive in their water quality risk management compared with other hospitals.

All previous studies on *Legionella* spp. in engineered water systems have either used standard culturing or a qPCR assay to detect *Legionella* spp, and none have screened for the presence of VBNC *Legionella* spp. In the present study, VFC+qPCR assay showed that all water and biofilm samples positive for *Legionella* spp./*L. pneumophila* gene marker also contained VBNC cells. The quantity of total *Legionella* spp. detected by qPCR assay was greater than VBNC cells, which clearly highlights that the hospital water system harbored both dead and VBNC *Legionella* spp. (Figure 1). Our findings suggest that the standard *Legionella* spp./*L. pneumophila* guidelines should include quantification of VBNC cells.

Currently, there is still much debate around the exact infective dose for *Legionella* spp. (Bartram et al., 2007). An analysis by Sikora et al. (2015) estimated that legionellosis outbreaks may occur sporadically when water is contaminated with 10^3 to 10^5 CFU/L

TABLE 4 Correlation between *Legionella* spp. and *Vermamoeba vermiformis* in hand basin and shower water samples.

	Spearman's rank correlation			
	Legionella DNA	VBNC *Legionella*	*L. pneumophila* DNA	VBNC *L. pneumophila*
Vermamoeba vermiformis	ρ = 0.5819 $p < 0.001$	ρ = 0.5833 $p < 0.001$	ρ = 0.5955 $p < 0.001$	ρ = 0.5826 $p < 0.001$

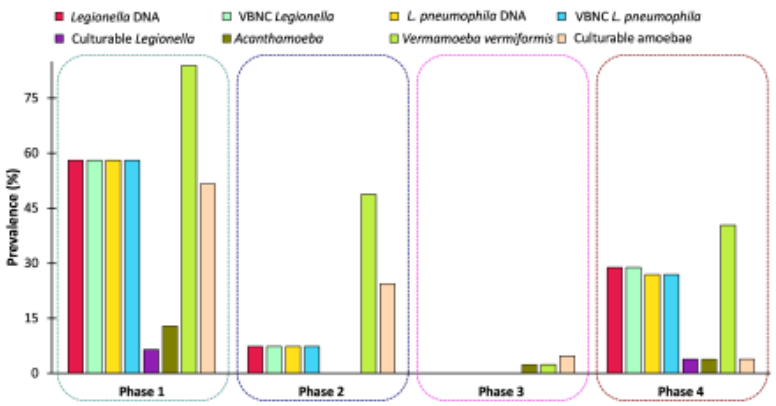

FIGURE 3
Relationship between intermittent water usage and the presence of *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents total duration (hours) of flushing events recorded for one-month prior to sampling. Flushing was categorized into low; 0 to < 2 hours, and high flow regime; ≥ 2 to 40 hours. Y-axis represents $\log_{10}$(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*.

and when *Legionella* spp. counts exceed 10^5 CFU/L an outbreak of legionellosis can occur (Sikora et al., 2015). However, these estimates are based on the number of culturable cells (CFU/L), so it is challenging to determine the relative risk associated with the concentrations of VBNC cells (GU/L). In contrast with culturable *Legionella* spp., VBNC cells infect with lower pathogenicity and take a longer time to infect amoebae (Nisar et al., 2023). Therefore, future research is needed to determine the infectious dose of VBNC *Legionella* spp. to understand the role of VBNC *Legionella* spp. in nosocomial infections and the public health risk posed by this concentration of VBNC *Legionella* spp. in engineered water systems.

In hospital water systems, *Legionella* infects and survives within protozoan hosts, including *Acanthamoeba* and *V. vermiformis* (Nisar et al., 2020a). In this study, it was identified that *V. vermiformis* (gene marker and culturable) was the most commonly identified amoebae associated with *Legionella* spp. prevalent in the water and biofilm samples (Table 4). This is supported by previous studies that have demonstrated *V. vermiformis* to be widely present in potable water (Kuiper et al., 2006; Nisar et al., 2020a). Similarly, microbiome analysis of potable water also showed *V. vermiformis* as the most prevalent protozoa (Delafont et al., 2013; Delafont et al., 2016). Water samples of dental units of Italian hospitals were found to be highly contaminated with *V. vermiformis* (60%) (Spagnolo et al., 2019). Similarly, a study conducted in hospitals of South Africa identified that 69% of samples were positive for *V. vermiformis* and 30.6% for *Acanthamoeba* (Muchesa et al., 2018). A recent study conducted in Australia examined water and biofilm samples from hospital and residential buildings showed the presence of FLA in 69% of the samples. It was also found that in all tested samples, *V. vermiformis* (55%) was the more frequently detected FLA (Nisar et al., 2022). In comparison with *Acanthamoeba*, *V. vermiformis* is more sensitive to disinfection treatments (Nisar et al., 2020a). Therefore, high levels of *V. vermiformis* could be attributed to decay and lower levels of residual chemical disinfectants in the hospital water system.

To our knowledge HPC loads have not been linked to any known legionellosis outbreak and the relationship between HPC levels and opportunistic premise plumbing pathogens is still unclear (Bartram et al., 2003). The SA Health, (2013) use HPC load as an indicator of water quality and recommend that if HPC load is ≥ 10^2 CFU/mL in warm water systems then the disinfection procedures for engineered water system should be considered. In this study it was found that the water samples with high HPC loads (≥ 5×10^3 CFU/L) contained high quantities of both *Legionella* spp. and *L. pneumophila* (Figure 2; Table 3). These results are in accordance with a previous study conducted on engineered water systems of residential buildings, which also showed a positive relationship between HPC levels and concentrations of *Legionella* spp. (Ley et al., 2020). Similarly, it was also found that samples with high HPC loads harbored high levels of *V. vermiformis* (Figure 2; Table 3). The relationship between bacteria and FLA consists of three major types of interactions i.e., mutualism, parasitism, and predation (Shi et al., 2021). Generally, FLA are considered natural predators of bacteria, which could account for the high levels of *V. vermiformis* observed in the presence of high levels of HPC (Rodriguez-Zaragoza et al., 2005). It was also identified that all samples positive for *Legionella* spp./*L. pneumophila* were also positive for FLA. Furthermore, Spearman's analysis demonstrated a strong positive correlation between *Legionella* spp./*L. pneumophila* and *V. vermiformis*. In engineered water systems, FLA exist in both trophozoite (metabolically active) and cyst (dormant) states (Zhang and Lu, 2021). The trophozoites support intracellular proliferation of *Legionella* spp. and transformation of VBNC *Legionella* spp. into a culturable state (Solomon et al., 2000; Shadrach et al., 2005; Berk et al., 2008; Watanabe et al., 2016; Boamah et al., 2017). Amoebae cysts protect intracellular *Legionella* spp. from prolonged chemical and physical disinfection treatments (Dobrowsky et al., 2016;

Boamah et al., 2017). The significant role amoebae play in *Legionella* spp. survival in potable water systems suggests that guidelines for the control of *Legionella* spp. must consider acceptable limits of amoeba within these systems as a measure to control *Legionella* spp. concentrations.

Water stagnation within building distribution systems promotes the accumulation of biomass, decay of chemical disinfectants, and alters the water quality (Bedard et al., 2018). Therefore, this study investigated the effect of temporary stagnation induced by intermittent flushing and water usage on *Legionella* spp., with a special focus on VBNC *Legionella* spp. It was found that an increase in temporary stagnation once a month prior to sampling significantly ($p < 0.01$) increased the quantity of total *Legionella* spp./*L. pneumophila* and VBNC *Legionella* spp./*L. pneumophila* population; however, increased stagnation one week prior to sampling was not associated with increased risk (Figure 3; Table 3). This supports guidelines that recommend routine flushing of outlets to manage *Legionella* spp. within engineered water systems (Enhealth, 2015). To our knowledge, this is first study in which the effect of temporary stagnation, HPC load, and *V. vermiformis* on VBNC *Legionella* spp./*L. pneumophila* in hospital water systems has been investigated. This study averaged water temperatures across one week or one month prior to sampling for both the hot and cold water pipelines/outlets. As a result water temperatures were more similar to each other than anticipated. This is likely to explain the lack of a statistically significant difference in *Legionella* concentrations associated with different temperatures. Future research with a larger dataset is needed to explore the temperature relationship further.

5 Conclusion

In building plumbing systems, temporary stagnation arising through intermittent usage causes water quality to deteriorate. This study identified that temporary stagnation for over a month promotes the persistence of VBNC *Legionella* spp./*L. pneumophila*. Similarly, FLA and heterotrophic bacteria present in this temporary stagnant environment positively interact with *Legionella* spp./*L. pneumophila*. Therefore, temporary stagnation, FLA and heterotrophic bacteria must be managed for the proper control and prevention of LD. This study also showed that the standard microbiological culture method used to detection *Legionella* spp. returned a false negative result for 88% of the VBNC *Legionella* spp. positive samples. As all samples positive for VBNC *Legionella* spp. were also qPCR positive, this suggests that qPCR may be a more appropriate detection method for routine surveillance. However, future research is needed to investigate the concentrations of VBNC *Legionella* spp. that pose a risk to public health to enable interpretation of these results to inform improved *Legionella* spp. guidelines.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

MN and HW conceived and designed research. MN performed the experiments. GB assisted in the flow cytometry. KR, MB, HW and RB provided technical assistance. JX and JH assisted in sampling and data collection. MN and HW drafted and edited the manuscript. HW, KR, MB, RB, GB, JX, and JH corrected and contributed to the manuscript. All authors approved of the final manuscript.

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Conflict of interest

Authors JH and JX are employed by Enware Pty Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1190631/full#supplementary-material

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Anexo 2. Capturas del artículo n°2 titulado: Environmental Monitoring of *Legionella* in Hospitals in the Campania Region: A 5-Year Study.

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Article

Environmental Monitoring of *Legionella* in Hospitals in the Campania Region: A 5-Year Study

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Abstract: *Legionella* is a pathogen that colonizes soils, freshwater, and building water systems. People who are most affected are those with immunodeficiencies, so it is necessary to monitor its presence in hospitals. The purpose of this study was to evaluate the presence of *Legionella* in water samples collected from hospitals in the Campania region, Southern Italy. A total of 3365 water samples were collected from January 2018 to December 2022 twice a year in hospital wards from taps and showers, tank bottoms, and air-treatment units. Microbiological analysis was conducted in accordance with the UNI EN ISO 11731:2017, and the correlations between the presence of *Legionella* and water temperature and residual chlorine were investigated. In total, 708 samples (21.0%) tested positive. The most represented species was *L. pneumophila* 2–14 (70.9%). The serogroups isolated were 1 (27.7%), 6 (24.5%), 8 (23.3%), 3 (18.9%), 5 (3.1%), and 10 (1.1%). Non-*pneumophila Legionella* spp. represented 1.4% of the total. Regarding temperature, the majority of *Legionella* positive samples were found in the temperature range of 26.0–40.9 °C. An influence of residual chlorine on the presence of the bacterium was observed, confirming that chlorine disinfection is effective for controlling contamination. The positivity for serogroups other than serogroup 1 suggested the need to continue environmental monitoring of *Legionella* and to focus on the clinical diagnosis of other serogroups.

Keywords: *Legionella*; environmental monitoring; Campania region; hospital; residual chlorine; temperature

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1. Introduction

Legionella is an aerobic, non-spore-forming, and Gram-negative pathogen [1], which was discovered in 1976 in Philadelphia following an outbreak of cases of pneumonia in a hotel [2]. Over 65 species belong to this genus [3], about 20 of which can cause disease in humans [4]. Moreover, *Legionella pneumophila* includes 15 serogroups, and serogroup 1 is the most dangerous to humans. Thus, the attention focused on this serogroup is high [4]. Furthermore, previous studies demonstrated human infections with serogroup 3 [5–7], serogroup 9 [7,8], and serogroup 6 [7,9].

Favourable factors to the growth of this bacterium are temperatures between 25 °C and 45 °C (but ranges between 5.7 °C and 63.0 °C can determine its survival); stagnant water [10], in which *Legionella* in amoebae copiously reproduces [11]; low flow [12]; pH values between 5.5 and 9.2; biofilm and protozoa [2]; inorganic elements (iron, zinc and potassium) and organic and inorganic compounds [13]; existence of water systems of old facilities [14]; dead branches in complex water structures [15]; low total chlorine levels [16]; and low free residual chlorine levels [17]. In addition, man-made disasters [18], natural disasters, flooding [19], water system interruptions, changes in disinfection methods of water systems, and water network breaks [11] are risk factors for *Legionella* growth.

Construction activities (such as demolition, repressurization, excavation, underground utility connections, commissioning at building opening, and water efficiency challenges) have been associated with healthcare-associated *Legionella* infections and deaths [20].

Hospitals need a water safety plan (WSP) to control *Legionella* proliferation, which includes the implementation of safety measures to ensure the water quality of facilities during demolition and construction activities and reduce the risk of exposure of patients [21]. Furthermore, hospitals need a WSP not only during construction or demolition activities. In fact, the World Health Organization (WHO) in 2004 recommended the creation of WSPs by all water suppliers: each of them should engage a group of water experts to assess the risks associated with water exposure, develop and implement strategies to prevent damage to public health, and evaluate the strategies' effectiveness [22]. Such a plan aims to minimize colonization of *Legionella* from the source of water supply to the devices in contact with users [23].

Chlorine-based disinfection is the most common active measure used worldwide, and in Italy, against *Legionella* growth and spread in building water systems [24,25]. Levels of free residual oxidant (FRO), within 0.20 ppm and 4.0 ppm in potable water, have been correlated with reduced risks of growth and spread of disease outbreaks [26].

The presence of *Legionella* can be detected in soil and water sources, such as showers, hot tubs, air-conditioning systems [3], cooling towers, whirlpools, baths, fountains, ice machines, medical equipment such as aerosols from respiratory devices, eye-wash stations, dental units [27], hot-water recirculation systems [28], hot- and cold-water systems, faucets [29], heater–cooler units, and heater units for cardiac procedures [30].

Legionella infection occurs through inhalation or aspiration of droplets released from contaminated water [3]. In addition, potting soil can also transmit the bacterium, but the mechanism still remains unknown [27]. People who are most affected by *Legionella* infections are smokers [31], alcohol abusers [32], males, those of advanced age [33], and people with previous diseases, such as acquired immunodeficiency syndrome, hematologic malignancy [34], and diabetes mellitus [35]. Consequently, it is necessary to monitor the presence of *Legionella* in hospitals as these are places where immunocompromised patients reside [36]. In healthcare settings, the presence of *Legionella* has been linked to contaminated water reservoirs, cooling towers and air-conditioning systems [37–39]. The occurrence of *Legionella* spp. in the water distribution systems of hospitals and healthcare facilities is a possible concern for hospital populations, due to the vulnerable nature of patients admitted to specific wards, including intensive care, hematology, cardiology, hemodialysis, and pulmonology [40].

The objective to be achieved in healthcare facilities is to minimize colonization by *Legionella* or, in the case of facilities housing immunocompromised individuals, its total absence (not detectable with the analytical method used) [41].

Legionella infection may have several consequences: it may cause Legionnaires' disease (LD) (a severe pneumonia), Pontiac fever (a flu-like condition), or it may remain asymptomatic. Because of the lack of specific symptoms and the absence of severity associated with this entity, Pontiac fever is often undiagnosed and under-notified [42].

There is a difference between patients who are hospitalized for LD [43] and others who acquire the infection in hospital due to susceptible medical conditions [44]. LD can be the cause of CAP (community-acquired pneumonia) and pneumonias that are acquired in situations other than travel, domestic environments, and hospitals, and it can be the cause of up to 30% of these types of pneumonias that require hospitalization [43]. *Legionella* infection can also be acquired in hospitals (hospital-associated infection): individuals most at risk from such an infection are those with prior situations that render them vulnerable, such as immunodeficiency, stem cell or organ transplantation, and obstructive pulmonary disease [44].

Legionella infections are becoming a public health problem due to their incidence and costs [45]. In order to prevent and control *Legionella* infections sourced from the colonization of water systems, many countries have developed guidelines or regulations. Several organizations, including the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE), the WHO, the Centers for Medicare and Medicaid Services (CMS), and the Centers for Disease Control and Prevention (CDC), recommend

the creation of water management programs aimed at preventing the growth and spread of *Legionella* [46].

In the COVID-19 era, it was reported that 20% of patients had a *Legionella* co-infection during hospitalization [47]. COVID-19 patients have an increased risk for both hospitalization and residual lung impairment [48].

In 2021, the number of Italian individuals affected by Legionellosis was 2726, and its incidence was 46.0 cases per million population [49]. The incidence increased compared to 2020, when the value was 34.3 cases per million population. Of the 2726 notified cases, 83.6% had a community origin, 9.4% were associated with travel, 3.7% had a nosocomial origin, 3.1% were associated with closed communities (nursing homes for elderly people, and healthcare or rehabilitation facilities), and 0.2% had another exposure (prison or communities). Infections of nosocomial origin increased from 68 to 102 cases from 2020 to 2021 [49]. However, *Legionella* infections are underestimated, and it is reported that less than 5% are diagnosed [50].

Legionellosis is a condition that can be avoided if the bacterium is not present in the environment, so monitoring and preventing its presence is important [51]. Environmental monitoring of *Legionella* is an approach performed on several occasions in hospitals in Italian regions by Deiana et al. [40], Ditommaso et al. [52], Vincenti et al. [53], De Giglio et al. [54], Laganà et al. [14], Arrigo et al. [55], Pasquarella et al. [56], and Torre et al. [57]; in non-hospital facilities by Totaro et al. [58,59], Sabatini et al. [60], and De Filippis et al. [61]; and in both hospital and non-hospital facilities by Felice et al. [62], Leoni et al. [63], and Mazzotta et al. [64]. Moreover, several studies were also conducted using air samples by Montagna et al. [65–67].

To our knowledge, very few studies performed environmental monitoring of *Legionella* in the Campania region, Southern Italy. In particular, a study by Torre et al. monitored the bacterium in 50 hospitals in the Campania region during the period 2008–2014 [39]. As the new Italian National Guidelines [41] were published in 2015, it was decided to conduct this study to assess the effectiveness of the application of the new Guidelines in preventing *Legionella* colonization in water systems. Thus, the aim of this study was to analyze hospital environmental monitoring of *Legionella* in the Campania region over the 5-year study period. In detail, the purposes of the study were (i) to evaluate the presence of *Legionella* in tested water samples; (ii) to estimate the prevalence of species and of individual *L. pneumophila* serogroups; and (iii) to assess the influence of several parameters, such as temperature and free residual chlorine, on the presence of this bacterium.

2. Materials and Methods

2.1. Study Area and Hospital Characteristics

The water sample collection was conducted from January 2018 to December 2022 in two provinces of the Campania region, Southern Italy (Naples and Caserta) (Figure 1). The samples were collected in hospitals which ask us to routinely perform *Legionella* testing every six months as part of WSPs as required by the Italian regulatory system for validation of a well-maintained building water system. The water samples were collected from 26 hospitals, all of which possessed the following characteristics: the presence of a single building, construction between 1970 and 1980, the presence of a maximum of five floors, and the presence of the number of beds served ranging between 20 and 200. These hospitals all have WSPs in place to respond to positive samples. We monitored 26 hospitals compared to the 50 hospitals in the study by Torre et al. [39], because some of the 50 hospitals were excluded since monitoring had not been carried out for all the years of the study, or these facilities were not comparable to the others (in terms of number of beds served, number of buildings, number of floors, construction period, or water treatment disinfection methods).

Water provided to the hospitals (which contains free chlorine as residual drinking water disinfectant) comes from the public supply system of the cities of Naples and Caserta, and it reaches the hospitals via a single pipeline. The hospital sites within the present study included buildings hosting patients considered at increased risk (Medicine, Pneumology,

Geriatrics, Surgeries, etc.) [41] over the study period, inclusive of the time during the COVID-19 pandemic.

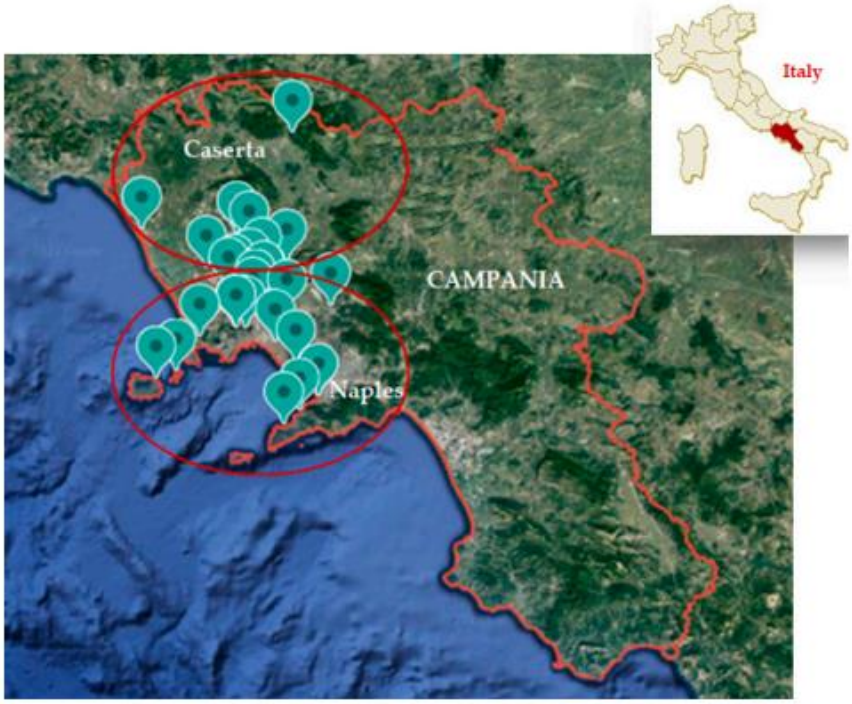

Figure 1. Study area map.

Samples were collected every six months, as required by the Italian National Guidelines for hospitals with this type of wards. If a sample tested positive, according to the Italian National Guidelines, the sampled point was decontaminated and re-sampled 1, 3, and 6 months later (these mandatory follow-up lab samples and corresponding data were excluded from the present study's analysis, as the purpose of the work was to monitor *Legionella* during routine monitoring activities). Therefore, we included the initial positive samples and excluded the follow-up samples. The water treatment disinfection methods used in these hospitals are based on the application of hypochlorite.

2.2. Sample Collection

We chose the sampling locations described in the Italian National Guidelines [41]. In addition, this study used similar sampling locations (i.e., faucets, showers, and tank bottoms) within the building premise plumbing system of the study by Torre et al. [39]. Our study, in addition to the previous sampling locations, also analyzed samples collected from the cold-water circuit, as mentioned in the new Guidelines.

In detail, for each hot-water system, as described in the Italian National Guidelines [41], the following sampling locations were carried out: supply, recirculation, and tank bottoms, with at least 3 representative points (furthest in the water distribution and coldest). Tank bottom refers to a hot- or cold-water storage tank. For each cold-water system, the following sampling locations were carried out: tank bottoms, with at least 2 representative points (furthest in the water distribution and hottest). In addition, water was collected from air-treatment units (ATUs). Following the observation of the positivity rate for *Legionella*, it was decided to increase the sampled points. The COVID-19 pandemic prompted us to further increase the number of samplings, and with its end, we decided to decrease the number of sampled points. Of the total of 3365 water samples, 2065 originated from

manual taps and showers (1485 from hot water and 580 from cold water), 780 from tank bottoms (in particular, 520 from hot water and 260 from cold water), and 520 from ATUs. The water samples were collected during the day (later in the morning, when the hospital was already in action). There were no point-of-use (POU) filters at any point of sampling.

According to the Italian National Guidelines, 1 L of water was collected by the laboratory staff in sterile polyethylene bottles enriched with 0.01% sodium thiosulfate to neutralize chlorine action [60]. All samples were collected without flushing and flaming or disinfecting at the point of discharge to simulate the common use of water, i.e., the exposure of a user. The water samples were univocally identified on a spreadsheet at the time of collection. At sampling, water temperature and residual chlorine were measured by our laboratory staff. Water temperature (expressed in °C) was obtained using a calibrated thermometer (TFA Digitales Einstichthermometer, TFA-Dostmann, GmbH & Co. KG, Wertheim-Reicholzheim, Germany), and free residual chlorine (expressed in mg/L) was monitored using a colorimetric diethyl-p-phenylenediamine (DPD) method (MQuant; Merck, Darmstadt, Germany). In detail, the test was based on a semi-quantitative measurement of free chlorine by visual comparison of the color of the measurement solution and a set of colors contained in a color card comparator.

The samples were transported, divided between hot- and cold-water samples at room temperature, and protected from light.

Calibrations (kits and equipment) and microbiological analysis were performed in our laboratory, which is accredited according to the ISO 17025 and periodically performs proficiency tests.

No samples were damaged during transport or showed a suspect appearance, such as different coloring or the presence of sediment or soil. Therefore, all samples collected were included and analyzed in the study.

2.3. Microbiological Analysis and Identification

The microbiological analysis was conducted within 2 hours after the collection of the samples, in accordance with the UNI EN ISO 11731:2017 [55,64]. Briefly, the samples were filtered on polycarbonate membrane filters with a pore size of 0.2 µm (Sartorius). The membrane was kept in 10 mL of the original water sample and vortexed. A total of 200 µL of water previously treated at (50 ± 1) °C for (30 ± 2) min. and the same volume of untreated water were inoculated on Petri plates containing *Legionella* Agar Base (Oxoid) medium and being supplemented with *Legionella* Growth Supplement (BCYE) (Oxoid) and *Legionella* Selective Supplement (GVPC) (Oxoid). The plates were incubated at (36 ± 2) °C with 2.5% CO_2 and under a humid atmosphere. After 10 days, the presence or absence of colonies was evaluated. The presumed *Legionella* colonies were cultured both on BCYE agar and BCYE agar without L-cysteine (BCYE-cys agar). The growth of colonies on the BCYE agar and not on the BCYE-cys agar suggested *Legionella* positivity of the samples. Determination of species and serogroups was conducted by the latex agglutination test (Oxoid) and the anti-*Legionella pneumophila* monovalent serum (Biogenetics). The colony counts were reported in terms of CFU (colony-forming units)/L.

The test results were sent to the hospitals.

2.4. Statistical Analysis

A descriptive statistical analysis was performed as previously described [39]. It included, for bacterial load values of positive samples, detection of geometric mean (Log_{10} CFU/L), standard deviation, median, percentile range, and interquartile range [39]. This analysis was conducted using Microsoft Excel.

Normality tests were conducted using the Shapiro–Francia test to check for data distribution. The non-parametric Mann–Whitney U test was used to determine the connections between the presence of *Legionella* and residual chlorine (expressed in mg/L) and water temperature (expressed in °C) [68]. The statistical results were interpreted at the level of significance $p < 0.05$. The χ^2 was calculated using Doornik–Hansen test. Multiple linear

regression analysis (MLRA) was used to confirm the results of the Mann–Whitney U test. The statistical calculations were performed using the STATA MP v14.0 statistical software program (College Station, TX, USA).

3. Results

3.1. Positivity of Analyzed Samples

During the 5-year study period, the laboratory analyzed a total of 3365 water samples. Among these samples, positivity for *Legionella* was detected in 708 samples, representing 21.0% of the total. The number of samples per year, along with the number of positive samples per year, is shown in Figure 2 and Table 1. Specifically, the number of positive samples decreased from 164 (34.2%) to 111 (14.7%).

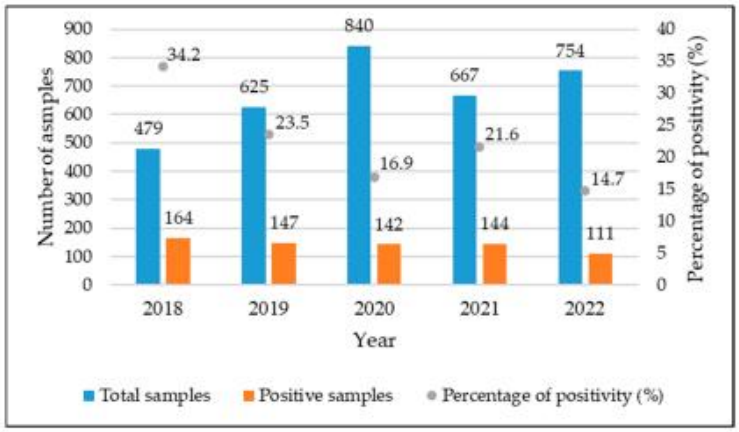

Figure 2. Number of collected samples, number of positive samples, and positivity percentages by sampling year.

Table 1. Summary of the total number of samples collected during the 5-year study period (2018–2022) with the number of positive samples and related percentages.

Sampling Year	Total Number of Samples	Number of Positive Samples (%)
2018	479	164 (34.2)
2019	625	147 (23.5)
2020	840	142 (16.9)
2021	667	144 (21.6)
2022	754	111 (14.7)

Table 2 shows the number of total samples, the number of positive samples, and the percentage for different sampling locations. Of the total number of cold-water samples (840), 37 (4.4%) tested positive. In particular, 11 originated from tank bottoms and 26 from taps and showers. In the hot-water samples, out of the 2005 total samples, 652 (32.5%) were positive. A total of 143 samples came from tank bottoms and 509 from taps and showers. In the ATU samples, a positivity rate of 3.7% was observed (19 positive samples out of a total of 520).

Table 2. Total samples, positive samples, and percentages for different sampling locations.

Tank Bottoms		Taps and Showers		ATUs
Cold	Hot	Cold	Hot	
N. (%)	N. (%)	N. (%)	N. (%)	N. (%)
11/260 (4.2%)	143/520 (27.5%)	26/580 (4.5%)	509/1485 (34.3%)	19/520 (3.7%)

3.2. Distribution of Species and Serogroups among Positive Samples

Figure 3 exhibits the percentage of positivity of *Legionella* species and serogroups in the years 2018–2022. In detail, *L. pneumophila* was the most represented (98.6% versus 1.4% of non-*pneumophila Legionella* spp.). Serogroups 2–14 had a positivity percentage of 70.9%, and serogroup 1 has a positivity percentage of 27.7%. Among serogroups 2–14, out of the total number of isolated species and serogroups, the most represented were serogroups 6 (24.5%), 3 (18.9%), and 8 (23.3%). Serogroups 5 and 10, on the other hand, were detected at a rate of 3.1% and 1.1%, respectively (Figure 3).

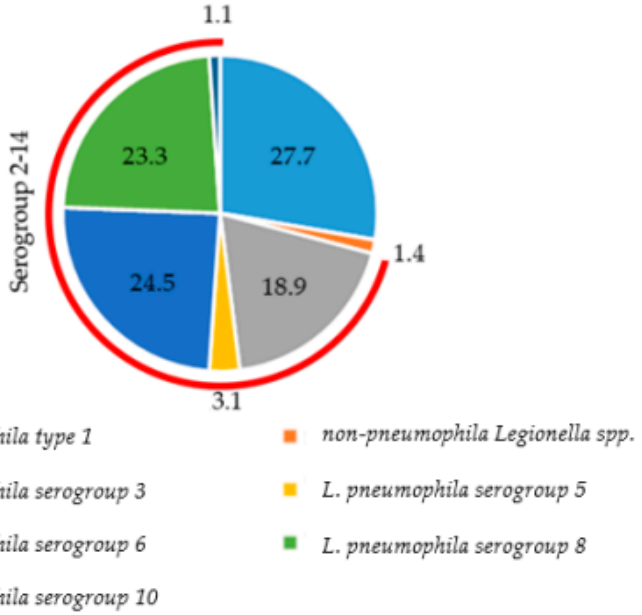

Figure 3. Percentage of positivity of each *Legionella* species and serogroup during the five-year study period (2018–2022).

In addition, in 27 samples of the 708 positive samples, the simultaneous presence of two species or serogroups was found. Particularly, out of these 27 samples, 22 samples tested positive for both serogroups 1 and 3 (3.1%), 1 sample tested positive for serogroups 1 and 6 (0.1%), 2 samples tested positive for serogroups 1 and 8 (0.3%), 1 sample tested positive for serogroups 1 and 10 (0.1%), and 1 sample tested positive for serogroup 5 and non-*pneumophila Legionella* spp. (0.1%).

3.3. Water Temperature and Residual Chlorine

Of the 3365 analyzed samples, 520 were taken from the ATUs in which the temperature and residual chlorine parameters were not carried out, while the remaining 2845 samples were divided into hot-water samples (≥26.0 °C, n. 2,005) and cold-water samples (≤25.8 °C, n. 840), with a mean temperature of 37.5 °C (range of 11.0–91.0 °C) for all the samples collected.

Table 3 shows different ranges of temperature and, for each interval, the number of analyzed samples, the number of positive samples, the minimum *Legionella* concentration, the maximum *Legionella* concentration, and the geometric mean of the positive samples. The majority of *Legionella* positive samples were found in the temperature ranges of 26.0–30.9 °C, 31.0–35.9 °C, and 36.0–40.9 °C (57.4%, 46.9%, and 48.8%). The percentage of positive samples decreased with increasing temperatures (from 48.8% for the temperature range of 36.0–40.9 °C to 19.4% for temperatures ≥56.0 °C).

Table 3. Number of analyzed samples, number of positive samples, percentage of positive samples, minimum and maximum values, and geometric mean of *Legionella* concentration of positive samples for different ranges of temperature.

	Temperature (°C)	Analyzed Samples	Positive Samples (%)	*Legionella* Concentration (Log_{10} CFU/L) Min	Max	Geometric Mean
Cold-water samples (n. 840)	≤20.9	382	19 (5.0%)	1.70	3.81	2.81
	21.0–25.9	458	18 (3.9%)	1.70	4.00	2.83
Hot-water samples (n. 2005)	26.0–30.9	54	31 (57.4%)	1.70	4.18	3.10
	31.0–35.9	179	84 (46.9%)	1.70	4.23	2.98
	36.0–40.9	240	117 (48.8%)	1.70	4.30	3.00
	41.0–45.9	558	170 (30.5%)	1.70	4.30	2.96
	46.0–50.9	549	153 (27.9%)	1.70	4.36	2.87
	51.0–55.9	296	72 (24.3%)	1.70	3.96	2.67
	≥56.0	129	25 (19.4%)	1.70	3.97	2.73

Regarding the bacterial concentration of the positive samples, a minimum value corresponding to 1.70 Log_{10} CFU/L was observed at all temperature ranges, while the highest maximum value was observed at the temperature range of 46.0–50.9 °C (4.36 Log_{10} CFU/L).

In addition, the minimum value of the geometric mean was 2.81 Log_{10} CFU/L in temperatures ≤20 °C, and it increased with increasing temperature, up to the range of 26.0–30.9 °C (3.10 Log_{10} CFU/L). For the temperature range of 41.0–45.9 °C and above, there was a decrease in the geometric mean value as temperature increased. At temperatures higher than 62 °C, no positivity for *Legionella* was observed.

3.4. Statistical Analysis

The results of the statistical analysis carried out to examine the bacterial load values of the positive samples are shown in Table 4. This table reports the number of total samples, the number of positive samples and percentage of positivity, the geometric mean (Log_{10} CFU/L), the standard deviation, the median, the percentile range, and the interquartile range. Moreover, the results show the normality of all data collected, and the Doornik–Hansen test for multivariate normality provides a $\chi^2 = 1.09 \times 10^5$.

The lowest *Legionella* concentration value recorded during the analysis was 1.70 Log_{10} CFU/L in all years, while the highest value was 4.36 Log_{10} CFU/L in 2019.

Table 4. Number of total samples, number of positive samples and percentage of positivity, geometric mean (Log_{10} CFU/L) of positive samples, and descriptive statistics. Descriptive statistical analysis was conducted for the positive samples (N = 708).

Year	Pos./Tot. (%)	Mean (±SD [1])	Min	25th Percentile	Median	75th Percentile	Max
2018	164/479 (34.2)	3.02 ± 0.72	1.70	2.54	3.12	3.63	4.28
2019	147/625 (23.5)	2.71 ± 0.80	1.70	2.00	2.60	3.35	4.36
2020	142/840 (16.9)	2.82 ± 0.74	1.70	2.18	2.81	3.38	4.30
2021	144/667 (21.6)	2.96 ± 0.73	1.70	2.40	3.02	3.62	4.23
2022	111/754 (14.7)	3.14 ± 0.58	1.70	2.78	3.20	3.62	4.04

[1] SD: standard deviation.

The results of the MRLA (Table 5) indicated that no statistically significances were recorded for the water samples' temperature (p-value = 0.526), in contrast with residual chlorine (p-value < 0.05).

Table 5. Results of linear multiple regression.

	Unstandardized Coefficients		Standardized Coefficients	
	T	Standard Error	*t*	*p*-Value
Dependent Variable: Bacterial Concentration (CFU/L)				
Water Temperature (°C)	0.0005	0.0008	0.63	0.526
Residual Chlorine (mg/L)	−0.4844	0.0782	−6.19	<0.05

N = 2845.

Furthermore, the MRLA results revealed the statistically significant correlation between residual chlorine and *Legionella* concentration, in which was negative, as suggested by the t-value (−6.19) and *p*-value (<0.05).

The boxplot regarding the influence of residual chlorine is shown in Figure 4.

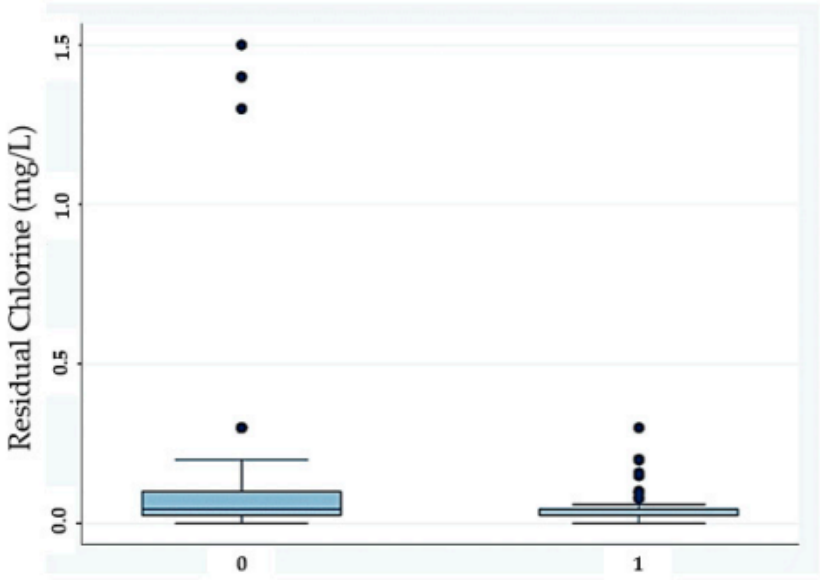

Figure 4. Boxplot showing the influence of chlorine residue (expressed in mg/L) on the presence of *Legionella*. 0 = No *Legionella* detected (N = 2156), and 1 = *Legionella* detected (N = 689).

4. Discussions

Legionella is a bacterium that colonizes soils, freshwater, and building water systems [51]. The aim of this paper was to analyze the prevalence of *Legionella* and its individual species and serogroups in hospitals of the Campania region over the period 2018–2022 and to assess the effectiveness of the Italian National Guidelines of 2015 in preventing *Legionella* colonization in water systems. In addition, the purpose was to test whether there was a relationship between the presence of *Legionella* with two variables, residual chlorine and water temperature.

In this study, we observed a 21.0% positivity rate. The positivity rate for *Legionella* decreased from 2018 (34.2%) to 2022 (14.7%). The number of positive samples gradually decreased, and this explains the decrease in the positivity percentage for this microorganism (Figure 2). Deiana et al. [40], on the other hand, monitored the presence of *Legionella* from 2010 to 2020 in a university hospital in the Sardinia region, Italy. Regarding the period 2018–2020, they also observed a decrease in the percentage of *Legionella* positivity in this region.

The authors posit that the decrease in *Legionella* positivity over the course of the study is likely attributed to the hospitals receiving the study's results and performing environmental hazard controls. The COVID-19 pandemic in 2020 and 2021 decreased attention to environmental *Legionella* prevention, leading to an increase in *Legionella* positivity in 2021. This increase was not observed in 2020 because the increase in hospitalization led to the need to implement hygiene rules (e.g., hand washing) [69], which could have promoted water flow and prevented stagnation. Stagnation is known to be responsible for the disappearance and poor stability of residual disinfectant and microbial growth of *Legionella* [70]. Moreover, the monitored hospitals were all in constant activity during the pandemic. The pandemic also directed our attention to new locations to monitor for *Legionella*: monitoring more locations in hospitals meant preventing *Legionella* infections in patients.

The percentage of positivity observed at the different sampling locations was 19.7% for tank bottoms, 25.9% for taps and showers, and 3.7% for ATUs. This study, in general, used similar sampling locations (i.e., faucets, showers, and tank bottoms) within the building premise plumbing system of the study by Torre et al. [39], conducted in the years 2008–2014 in hospitals in the Campania region, which showed a percentage of positivity of 27.2% for tank bottoms, 31.9% for taps and showers, and 4.0% for ATUs. The authors supposed that this decrease could be explained by the publication of the Italian National Guidelines in 2015 and the implementation of a WSP by hospitals, which resulted in the collaboration of different personnel (laboratory staff, technical office, water disinfection staff, etc.) in order to ensure water safety. In addition, the highest percentage of *Legionella* detection was observed in taps and showers, which are a source of exposure for patients (taps and showers can generate aerosols containing the bacterium that can be inhaled) [71].

Out of a total of 840 cold-water samples (temperature range of 11.0–25.9 °C), 37 (4.4%) tested positive. The presence of *Legionella* in cold-water samples was also found in the study by Arvand et al., 2011 [72], who detected a percentage of positivity of 40% in cold-water samples (temperature range of 7–29 °C) [72]. Our results are not surprising, since out of the total number of cold-water samples taken (840), 458 (54.5%) showed a temperature between 21.0 and 25.9 °C, which are values near the range of temperatures that can create favorable conditions for *Legionella* multiplication.

The results indicated that *L. pneumophila* was the most isolated species. The serogroups 2–14 showed a positivity rate of 70.8% for the positive samples, which was similar to that found in Turkish hospitals (70.8%) by Yilmaz and Orhan [73], in Italian non-hospital facilities (71.7%) by Sabatini et al. [60], in hospital samples (68.7%) collected by Deiana et al. [40], and in Polish non-hospital samples (64.3%) collected by Stojek and Dutkiewicz [74].

Regarding the individual serogroups, the highest percentage of positivity was found for serogroup 1. This finding is congruent with what is found in clinical diagnosis, according to which the *L. pneumophila* type 1 is the serogroup that most commonly affects humans [7,75,76]. Amemura-Maekawa et al., in fact, isolated an 80.2% percentage of serogroup 1-positive samples in Japanese clinical specimens collected from 1980 to 2008 [77].

Regarding serogroups 2–14, the presence of serogroups different from serogroup 1 (6, 8, and 3) was the same as found previously [39]. Serogroups 3 and 6 were also among the serogroups isolated from humans in Italy during 1987 and 2009 [78], so these results had correspondence with what was previously reported in humans. These findings were also in agreement with the results determined by Perola et al., who, through a genotyping analysis, found an association between the isolates from two *Legionella* serogroup 5-positive patients residing for several days in a hospital and the environmental isolates from the hospital's water supply [79].

Serogroups 3 and 6 were found in environmental water samples in several studies [54,80–83]. Particularly, serogroup 3 was isolated in hospitals in Iran [84], while Pignato et al. revealed the prevalence of this serogroup in Italian hospitals, which was sometimes associated with serogroup 6 [85]. Isolated serogroup 8 was reported by De Giglio et al. [54], Papadakis et al. [83], and Sakhaee et al. [84].

The presence of serogroups different from serogroup 1 suggests that attention should be focused on the clinical diagnosis of other serogroups, considering that the most widely used test for *Legionella* diagnosis in human specimens (the urinary antigen test) shows a low sensitivity for non-*L. pneumophila* type 1, resulting in underreporting of *Legionella* infections [76,86].

Regarding temperature, in our study, we observed that more hot-water samples were positive than cold-water samples (32.5% vs. 4.4%). This finding is consistent with that observed in the work by Stojek et al. conducted in Poland, who observed a higher frequency of *Legionella* isolation in hot-water samples (88.6%) [87]. In addition, the highest percentages of positive samples were found at temperatures ranging from 26.0 to 40.9 °C, which are temperatures corresponding to the optimal growth range of *Legionella*. For temperatures above this range, the percentage decreased. With regard to the bacterium's concentrations, the highest values were found at a temperature range of 46.0–50.9 °C, and above this temperature range, these values decreased. In the study by Boppe et al. conducted in a hospital where the correlation between the concentration of *L. pneumophila* and the maximum water temperature at the point of use was investigated, a significant decrease in the bacterium's concentrations was observed at temperatures ≥55 °C [88].

Our analysis revealed a *Legionella* concentration minimum value of 1.70 Log_{10} CFU/L in all years, and the highest value of 4.36 Log_{10} CFU/L was observed in 2019. In the study by Torre et al., the minimum and maximum values observed were 2.00 and 7.45 Log_{10} CFU/L [39]. On the other hand, in the study by Girolamini et al., the minimum and maximum concentration values observed in a hospital in the Emilia-Romagna region, Italy, which water was treated with a biocide, were between <1.70 and 5.80 Log_{10} CFU/L [89].

The Italian National Guidelines for the prevention and control of Legionellosis indicate the types of interventions required to be conducted in healthcare facilities, depending on the positivity rate, the *Legionella* concentration (expressed in CFU/L), and the presence or absence of clinical cases [41].

In addition, procedures must be performed to obtain non-detection of *Legionella* in air-treatment systems and water in wards housing profoundly immunocompromised and very high-risk patients (transplant centers, oncology, and hematology) and in the water for tank births.

If a sample tested positive, according to the Italian National Guidelines, the sampled point is decontaminated and re-sampled one, three, and six months later (these mandatory follow-up lab samples and corresponding data were excluded from the present study's analysis).

Regarding the trends of *Legionella* concentration found, this study revealed a statistically significant negative correlation with residual chlorine. This finding was in agreement with D'Alessandro et al. [90], who observed a statistically significant reduction in positive samples following the increase in free chlorine in water. Furthermore, Totaro et al. found a moderate relationship between the presence of *Legionella* and a decrease in total chlorine concentration [58]. The same result was reported by Masaka et al., who found a weak statistically significant negative correlation with both *L. pneumophila* serogroups 1 and 2–14 [91]. A statistically significant negative correlation between *Legionella* concentration and total chlorine residual concentration was reported by Zhang et al. [92]. Moreover, in their study, Rafiee et al. argued that there was proportionality between residual chlorine content and the presence of *Legionella* [93]. The mechanism by which chlorine combats the presence of *Legionella* involves it interaction with the pathogen's cell membrane [94]. This results in a dispersion of the cell's macromolecules and subsequent modification of the cell's chemical, physical, and biological processes [94].

Furthermore, no statistically significant correlation was found with water temperature, as reported by Masaka et al. [91] and Pierre et al. [46], and in contrast to the evidence found by Rakić et al. [68], who found a correlation with *L. pneumophila*. In addition, De Giglio et al. observed a weak correlation between *Legionella* concentrations and water temperature [95].

In summary, the results of the study confirm that chlorine disinfection is an effective method to control *Legionella*; this is in accordance with the results reported by Paranjape et al., who found that continuous application of chlorine inhibits the presence of *Legionella* in cooling towers, and by Orsi et al., who found that shock and continuous hyperchlorination significantly reduce the number of *Legionella* positive samples [16,96].

5. Limitations

The limitations of this study were that sampling covered only two provinces of the Campania region in the South of Italy. Thus, in the future, it would be interesting to expand the study to other provinces in order to have a complete overview of the situation in the study area. Moreover, the sampling covered only one type of facility. Consequently, a future goal could be to expand the analysis to other types of facilities, such as recreational centers, tourist sites, military bases, schools, and others, since it is possible to identify the bacterium in these structures [62]. In addition, only two variables were analyzed to evaluate the relationship with the presence of *Legionella* (water temperature and residual chlorine). Furthermore, we did not collect information about cases of *Legionella* infections in the monitored hospitals, and it would be interesting to investigate this aspect in future studies and assess whether there is a correlation between these two variables, as performed in the study by Perola et al. [79].

Finally, the circumstance that, in a high percentage of samples (79.0%), *Legionella* is not detected by the culture method does not mean that all samples are really negative for *Legionella*. There may be a percentage of samples that have viable but non-culturable (VBNC) *Legionella*, and the culture method cannot detect them. VBNC is a state of *Legionella* that can be established under unfavorable environmental conditions and retain virulence. Under favorable conditions and in free-living amoebae, the bacterium can resuscitate and become pathogenic again [97]. For example, a study conducted from 2010 to 2015 in an Italian university hospital subjected to continuous monochloramine treatment determined that 34.5% of the negative samples, as determined by the culture method, tested positive for the VBNC state. In addition, 18% of 22 tested samples were positive based on the resuscitation test [97]. This situation could occur in some of our negative samples. Future goals will include testing for the VBNC state.

This paper could be a starting point for future analyses, given the importance of environmental monitoring and the number of studies monitoring this bacterium in the Campania region in South of Italy.

6. Conclusions

Our environmental monitoring revealed bacterial contamination of water in this region, even though its presence decreased in percentage positivity from 2018 to 2022. Despite this decline, attention should not be reduced, especially in facilities housing immunocompromised individuals who are more susceptible to *Legionella* infection. The most represented species was confirmed to be *L. pneumophila*. Serogroups 2–14 were found at a high rate. Regarding total serogroups, the most represented was serogroup 1, followed by serogroups 6, 3, and 8. These results suggest the need for continuous environmental monitoring of *Legionella* and highlight the importance of focusing on the clinical diagnosis of serogroups different from serogroup 1 as well, since the most widely used test on human samples shows a high sensitivity only for this serogroup.

The highest concentration of positive samples was observed in the temperature range corresponding to the optimal growth temperature of the bacterium. In addition, the negative correlation between residual chlorine and the presence of *Legionella* confirmed that chlorine disinfection is an effective method for preventing *Legionella* contamination.

The implementation of a WSP, through the collaboration of different groups of professionals, is one of the main approaches to control *Legionella* contamination in hospitals and prevent infections.

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Anexo 3. Capturas del artículo n°3 titulado: Detection of Legionella pneumophila in the Water Samples of Food Industries and Hospitals in Bangladesh.

MICROBIAL BIOACTIVES RESEARCH

Detection of *Legionella pneumophila* in the Water Samples of Food Industries and Hospitals in Bangladesh

Nazmun Naher[1], Sangita Ahmed[1]*, and Md. Latiful Bari[2]

Abstract

Background: The human pathogen *Legionella pneumophila* causes a serious pneumonia-like respiratory disease called Legionnaires' disease, mainly in elderly and immunocompromised individuals. This pathogen can be found in the water distribution systems of large constructions with cooling towers which is a common phenomenon at present in Bangladesh due to its rapid economic growth. But there is a dearth of information on the incidence of *Legionella* in Bangladesh. Therefore, the current study aimed to investigate the presence of *Legionella pneumophila* in hospital and industrial water distribution systems in Dhaka, Bangladesh. Methods: A total of 114 water samples collected from two hospitals and five food industries were inoculated on the *Legionella*-specific medium Buffer Charcoal Yeast Extract (BCYE) agar medium before and after the treatment with acid, heat, or a combination of both. Samples producing *Legionella*-like colonies on BCYE agar medium were screened by *Legionella* Latex Test Kit, and the metagenomic DNAs obtained from these samples were analyzed by PCR using *L. pneumophila*-specific 16S rRNA primers. Results: Among 114 samples, *Legionella*-like colonies were observed in 30 water samples which demonstrated no agglutination in the Latex agglutination test. PCR analysis showed the presence of *L. pneumophila* in seven water samples, four in the potable water, chiller water, and cooling tower water of two different food industries, and three in ICU tap water, cooling tower water, and Fan Coil Units of two different hospitals. Sequence analysis of amplicons revealed that all seven sequences had 100% similarity with *L. pneumophila*. Conclusion: The presence of *L. pneumophila* in the water samples of local hospitals and food industries indicates that these habitats might serve as a potential site for Legionnaires' infection in Bangladesh. The results also showed that PCR, contrary to the conventional culture methods, could be more efficient and rapid in the identification of *L. pneumophila*.

Keywords: Legionnaires' disease; Water distribution system; *Legionella pneumophila*; PCR-based detection.

Abbreviations: BLASTN, Basic Local Alignment Search Tool; DNA, Deoxyribonucleic acid; ICU, Intensive Care Unit; µl, microliter; LPFP, *Legionella pneumophila* specific forward primer; LPRP, *Legionella pneumophila* specific reverse primer; NCBI, National Center for Biotechnology Information; ng, nanogram; PCR, Polymerase Chain Reaction; rpm, revolutions per minute; VBNC, Viable but nonculturable.

Introduction

Legionella pneumophila is a Gram-negative respiratory pathogen that causes pneumonia, with a mortality rate of 10% for elderly and immunocompromised patients (Alarcon Falconi *et al*., 2018). This pathogen is fastidious and ubiquitous in aquatic environments although its presence is at a very low or undetectable level in natural and artificial environments as well as buildings and mechanical equipment (WHO 2002, ASHRAE 2000). But the incidence of legionellosis, a serious type

Significance | PCR-based rapid detection of slow-growing *Legionella pneumophila*

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nocompromised patients (Alarcon Falconi *et al.*, 2018). This pathogen is fastidious and ubiquitous in aquatic environments although its presence is at a very low or undetectable level in natural and artificial environments as well as buildings and mechanical equipment (WHO 2002, ASHRAE 2000). But the incidence of legionellosis, a serious type of pneumonia, has increased significantly in recent years due to the extended use of water in large technical systems like hospitals, hotels, and industries (Falkinham, 2020; Tercělj-Zorman *et al.*, 2004; Weiss *el al.*, 2017). The potential risk sites in these facilities are premise plumbing systems, cooling towers, evaporative condensers, and hot and cold water systems with a temperature range of 20-45°C (Moens 2002). In these facilities, the bacteria attach, settle, grow and multiply in high numbers through biofilms (Pereira *et al.*, 2021) and might cause infection when susceptible individuals inhale the aerosol from the water distribution systems (Buse*et al.*, 2012). Therefore, the disease is a serious public health concern (Flannery*et al.*, 2006), and considering the threat to public health of *L. pneumophila*, the World Health Organization (WHO) and the U.S. Centers for Disease Control and Prevention (CDC) emphasize routine monitoring of this pathogen (CDC, 2005; Parr *et al.*, 2015).

There are quite a very few studies on the incidence of *legionellosis* in Bangladesh although cases of atypical pneumonia are being reported continuously throughout the world (Erdoğan and Arslan, 2013; Matsumoto *et al.*, 2006; Stamm and Stankewicz, 2022). The detection of *Legionella* was reported from natural water (Haque *et al.*, 2016; Vasanthabharathi and Jayalakshmi, 2018), hospital tap water, hotel, and clinical samples (Jonas *et al.*, 1995). In Bangladesh, the number of large industries and big hospitals is in increasing trend requiring the installation of air conditioners, cooling towers, hot and cold water systems, and decorative fountains at their premises. It is imperative to study whether the water distribution systems of these developments serve as the habitats for *L. pneumophila,* as the ambient weather condition of Bangladesh, with temperatures ranging from 20–45°C is favorable for the proliferation of this pathogen. Therefore, this study aimed to investigate the presence of *L. pneumophila* in water samples of different food industries and hospitals in Bangladesh.

Methodology

Sample collection

A total of 114 water samples were collected from two hospitals and five different food industries in Dhaka city from June 2019 to November 2019. Water samples were collected from 14 different points of two hospitals and five food industries, respectively (Table 1). Samples were collected in a sterile, non-transparent plastic bottle, maintained at average temperature, and immediately transported to the laboratory within 2 hours. For chlorine treated sample, 0.5 ml of 0.1N sodium thiosulfate was added to each 1.0 liter of water sample to neutralize the chlorine. The collected water samples were cultured following the detection method described in water quality-Enumeration of *Legionella* ISO 11731:2017.

Detection of *Legionella* using a culture-based method

Each water sample was filtered and concentrated by pouring the sample into a sterile 47 mm funnel assembly containing 0.45 µm polycarbonate filters. The filter was taken off aseptically and inserted into 5 ml of sterile water, and the tube was then vortexed for one minute to loosen the bacteria into the water. This concentrate (5ml) was cultured on Buffered Charcoal Yeast Extract agar medium (Difco, Germany) either directly (untreated) or after acid treatment (KCl–HCl solution, pH- 2.2), heat treatment (30 min at 50 °C) or combined acid and heat treatment. The plates were incubated at 35 °C in a 2.5% CO_2 incubator and were examined daily for up to seven days for the growth of *Legionella*. Presumptive *Legionella* colonies were identified based on colony morphology, and colonies that appeared round, glistening, and convex with frosted glass appearance (CDC, 2005) were subjected to a latex agglutination test by *Legionella* Latex Test Kit (LK04-Hi, Himedia) as per the manufacturer's instructions.

Isolation of total DNA

One liter of water was filtered with a 0.22 µ membrane filter, and the filter was washed with 10 ml of sterile deionized water. The wash-off water containing the retentate of the filter was centrifuged at 13000 rpm for 5 min, and the pellet was resuspended in 0.1 ml of sterile deionized water. Following incubation at 100 °C for 5-10 minutes in a water bath, the tube was immediately chilled on ice for 30 minutes. The suspension was then centrifuged for 5 minutes at 12000×g at 4 °C, and the supernatant containing the resulting metagenomic DNA was transferred to a new sterile microfuge tube and stored at -20 °C for further use.

Legionella pneumophila-specific 16SrRNA PCR

Metagenomic DNAs from the water samples were used to amplify the *Legionella*-specific 16SrRNA gene fragment following the method described by Jonas *et al.*, 1995. The primers used in these PCR reactions were LPFP: (5'–AGGGTTGATAGGTTAAGAGC-3'); and LPRP: (5'–CCAACAGCTAGTTGACATCG-3').

PCR was performed in a final reaction volume of 25 µl containing 12.5 µl of master mix (OneTaq quick load 2×Master mix, New England Biolabs), 0.5 µl of each forward and reverse primer, 2 µl of metagenomic DNA, and 9.5 µl of nuclease-free water. Amplification was carried out in a Thermal Cycler (Veriti, Applied Biosystems, USA) with initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1.5 min, annealing at 57 °C for 1.5 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. After amplification, the PCR products were processed for gel documentation and stored at -20 °C for further use.

Table 1. Collection of water samples from Hospital and Food industry water systems at Dhaka city.

Sources of water samples				
Sl. no.	**From Hospital**	**No. of samples**	**From Food industry**	**No. of samples**
1.	ICU Tap water	2	Reservoir water / Holding Tank	10
2.	Reservoir water / Holding Tank	4	Cooling Tower water	35
3.	Reverse Osmosis Dialysis water	3	Softener water	10
4.	Oxygen Flowmeter water	2	Potable water/ Drinking water	5
5.	Sterilize water for utensils	2	Bottle washing water	5
6.	Fan Coil Unit water	1	Hot Water Tank/ Preheated water	10
7.	ICU drinking water	2	Chiller water	5
8.	Cooling Tower water	1	Air condition water	10
9.	Softener water	1		
10.	Inlet water	1		
11.	Air Handling water	1		
12.	Potable water/ Drinking water	1		
13.	Hot Water Tank/ Preheated water	1		
14.	Chiller water	2		

Figure 1. Growth on BCYE agar media- a) *Legionella pneumophila* ATCC 33152, b) sample with presumptive growth of *Legionella*, c) sample with no growth of *Legionella* like colony (right)

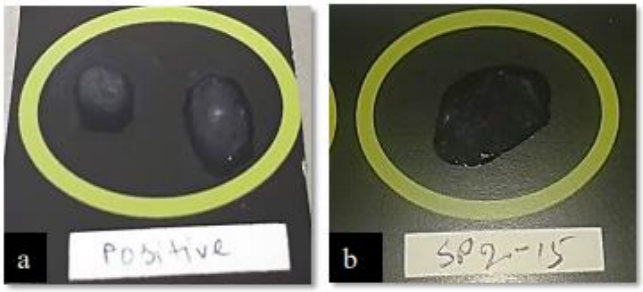

Figure 2. Observation on the latex agglutination assay. (a) Positive agglutination of *Legionella pneumophila* ATCC culture and (b) No agglutination for presumptive isolates.

The amplified DNA fragments were subject to horizontal gel electrophoresis in a 1.5% agarose gel slab and the gel was stained with ethidium bromide. The gel was visualized in a gel documentation system (Infinity Vilber Lourmat, France) after destaining. With strict adherence to the manufacturer's instructions, a PCR cleaning kit (Favorgen, Taiwan) was used to clean the amplified PCR products, and about 0.5-100 ng of the purified product was sent to Macrogen (Korea) for sequencing. The sequences of 16S rRNA gene fragments were submitted to the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) GenBank and the blast-n analysis was performed for identification (Shokraei *et al.*, 2019). A maximum likelihood phylogenetic tree was constructed based on these sequences using Molecular Evolutionary Genetics Analysis (MEGA) software version 11.

Results

Detection of *Legionella pneumophila* using culture method

A total of 114 water samples, treated with acid or heat or a combination as well as untreated water, were inoculated on Buffered Charcoal Yeast Extract Agar medium (BCYE) and round, glistening, convex, frosted glass colonies (typical characteristics for *Legionella* as described in the Procedures for the recovery of *Legionella* from the environment, CDC 2005), were isolated from 30 samples (Fig. 1).

Detection of *Legionella* by Latex agglutination kit

In the Latex agglutination assay, none of the presumptive *Legionella* isolates showed agglutination, while the positive control showed direct agglutination (Fig. 2).

Detection of *Legionella pneumophila* by Polymerase Chain Reaction

Detection of a 386-bp amplicon by PCR is considered a positive result for *Legionella* (Cloud *et al.*, 2000). Among the 30 samples tested, the *Legionella pneumophila*-specific PCR product was detected in the 7 samples (Fig. 3). A comparison among the sequence of these PCR products in the GenBank database of the NCBI showed 100% homology of all seven isolates with *L. pneumophila*. The isolates were designated with accession numbers by NCBI GenBank after necessary verifications, which are MZ102255, MZ102256, MZ102257, MZ102258, ON924465, ON924466, and ON924467 (Table 2). Three isolates were obtained from hospital ICU tap water, cooling tower water, and Fan Coil Unit water of two different hospitals, while the remaining four were obtained from food industry cooling tower water, potable water, and chiller water.

A maximum likelihood tree was constructed based on the LP16S rRNA sequences of the seven *L. pneumophila* (Fig. 4). The 16S rRNA sequences of three reference strains of *L. pneumophila* obtained from the NCBI database were included in the analysis. The phylogenetic analysis demonstrates proximity among the sequences of the seven *L. pneumophila* with those available in the database, including the ATCC strain MZ59157.1. The sequences were distributed in two different lineages with divergences from a single root.

Discussion

Legionellosis is not a commonly reported disease in Bangladesh which might be because of overwhelmed medical sectors with other common diseases, lack of available, cost-effective diagnosis methods, and lack of awareness about the disease among the people. There are also only a few scientific reports on *L. pneumophila* in clinical trachea samples in Bangladesh (Jahan *et al.*, 2015). Although the reports of atypical pneumonia are increasing day by day, the role of *L. pneumophila* in this connection is rarely investigated here in Bangladesh (Marchello *et al.*, 2016; Matsumoto et al., 2006). Therefore, this study was designed to investigate the prevalence of *L. pneumophila* in water samples in Bangladesh, initially focusing on the hospital and food industry, which holds potential sites for the growth of this pathogen. Screening of 114 water samples collected from 14 sites of two hospitals and five food industries revealed the presence of seven *L. pneumophila*. To the best of our knowledge, this is the first report on detecting *L. pneumophila* from the industrial water system in Bangladesh.

The culture method using the selective BCYE agar medium was considered the gold standard for detecting *L. pneumophila*. However, we did not find any *L. pneumophila* using the selective growth medium in the current study. Borges *et al.*, (2012) reported a similar observation where they found *Legionella*-like colonies on BCYE agar and none was *Legionella*, as further revealed by 16SrRNA sequencing. These reports suggest that the presence of other microorganisms or disinfectants in a water sample inhibits the growth of *Legionella* sp. on the BCYE medium (Gilpin and Gilpin, 2014). The presence of *L. pneumophila* in a viable but non-cultural (VBNC) state in the environment also interferes with its detection using a culture-based method (Ahmadrajabi *et al.*, 2016). The same might be the case for the current study, as in Bangladesh, disinfectants are regularly used to maintain water quality, and the presence of a large number of heterotrophic bacteria is also typical in water samples (Islam *et al.*, 2010; Islam *et al.*, 2020 ; Mahbub *et al.*, 2011; Shahidul *et al.*, 2014). The *L. pneumophila* ATCC33512 showed confluent growth on the BCYE medium in the current study, possibly because it was a pure culture and did not have any competitive flora or inhibitory factors. Borges *et al.*, (2012) reported the growth of the members of *Chitinophagaceae* on the BCYE agar media with typical gray colonies like *L. pneumophila*. It might be worthwhile to investigate whether the isolates that showed similar colony morphology on BCYE medium in the current study belong to *Chitinophagaceae*, which might have interfered with the study.

Table 2. Detection of *Legionella pneumophila* by PCR

Sources of water sample	Number of *L. pneumophila* detected by PCR	GeneBank accession number
Food industry		
Cooling tower water	2	MZ102257 MZ102258
Potable water	1	ON924467
Chiller water	1	ON924466
Hospital		
ICU tap water	1	MZ102255
Cooling tower water	1	MZ102256
Fan Coil Unit water	1	ON924465

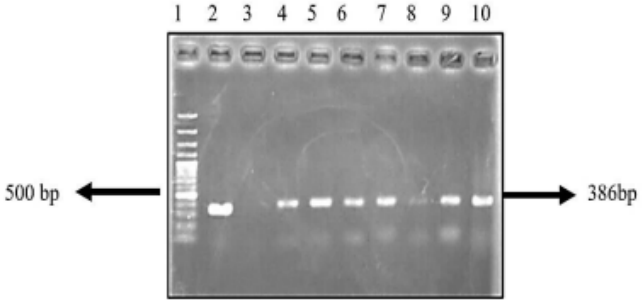

Figure 3. **Detection of *Legionella pneumophila* (LP) specific 16srRNA gene (386bp) in water samples.** (Lane 1: 100 bp DNA ladder, Lane 2: Positive control *Legionella pneumophila* ATCC33512, Lane 3: Negative control, Lane 4-7: Cooling tower water, Potable water, Chiller water samples from food industry, Lane 8-10: Fan Coil Unit water, ICU tap water, Cooling tower water from hospital).

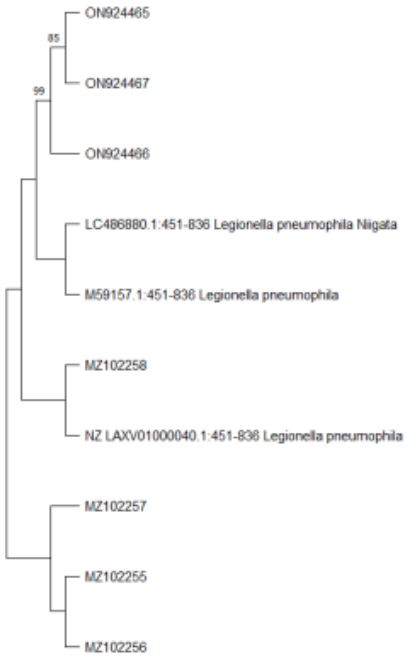

Figure 4. **A maximum likelihood phylogenetic tree was constructed based on LP16SrRNA sequences.** Sequencings were Aligned by ClustalW algorithm Bootstrap method using Molecular Evolutionary Genetics Analysis (MEGA) software version 11. The values above nodes indicate the bootstrap values. Bootstrap values below 75 are not displayed.

In contrast to the culture-based method, the *Legionella*-specific PCR assay, targeting the 16S rRNA gene of *L. pneumophila* was found to be more sensitive for detecting this pathogen, as seven *L. pneumophila* have been detected in the current study using the PCR assay. This finding is in agreement with other studies where compared to the culture-based methods, PCR assay exhibited higher efficacy in the detection of *L. pneumophila*, even those in the VBNC state (Ahmadrajabi *et al.*, 2016; Shishir and Hoq, 2020; Rafiee *et al.*, 2014; Wellinghausen *et al.*, 2001). This finding also emphasizes formulating a new growth medium, which would be more selective and sensitive for detecting this pathogen from an environmental sample.

In the current study, 43% of *L. pneumophila* were detected in cooling tower water samples of one hospital and two food industries, which is in alignment with global data of cooling tower systems being the major reservoir of *Legionella* (Ishimatsu *et al.*, 2001; Lam *et al.*, 2011; Pagnier *et al.*, 2009; Van den Hoek *et al.*, 2006). *L. pneumophila* was also detected in the tap water of the ICU and Fan Coil Unit of the hospital. These data, therefore, indicate that the water distribution systems of hospitals in Bangladesh might be a credible source of transmission of this pathogen among admitted patients. However, as there are no reported cases of Legionnaires' disease in Bangladesh, it is difficult to establish a link between the presence of this pathogen in hospitals and the actual disease. However, atypical pneumonia should be investigated extensively for the etiological agents. To investigate any such possibility, implementing a routine diagnosis of *L. pneumophila* in hospital-acquired pneumonia patients in Bangladesh and regular surveillance of the hospital water system is essential.

Phylogenetic analysis of the LP16SrRNA sequences displays proximity among the sequences of the seven *L. pneumophila* obtained in the current study with those available in the database. However, no specific association was observed between the sequences and sample collection sites. The sequence of MZ102258, detected in a food industry cooling tower, was homologous to the reference sequence NZ LAXV01000040.1, which was detected in a cooling tower in China, suggesting a possible link. A more extensive study of the genome sequences of this pathogen is crucial to identify the route of transmission of *L. pneumophila* in Bangladesh.

This study demonstrates that water distribution systems of hospital and food industries are colonized by *L. pneumophila* in Bangladesh and present a possible threat to the people, which emphasizes regular surveillance and decontamination of water supply in these premises. Observations from the study form the basis of more comprehensive research on the prevalence of *L. pneumophila* in other potential sources in Bangladesh.

Conclusion

From the present investigation, it can be concluded that *Legionella pneumophila* might be prevalent in the water distribution systems in Bangladesh, which needs further exploration. The data from the current study suggest that the PCR method, instead of the conventional culture-based technique, would be more effective and rapid for the detection of this pathogen from water samples.

Author Contribution

The manuscript has been read and approved by all authors. The research work has been conceptualized and supervised by Professor SA and MLB. NN designed and conducted the research work, analyzed the data, and was involved in writing and editing the script. The corresponding author is the sole contact for the editorial process. She is responsible for communicating with other authors about progress, submission of revisions, and final approval of proofs.

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Competing financial interests

The authors declare that they have no competing financial interests.

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Anexo 4. Capturas del artículo n°4 titulado: A Study on The Presence of Legionella pneumophila in Hospital Water Samples from Eastern Turkey.

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Original Article / Özgün Araştırma

A Study on The Presence of Legionella pneumophila in Hospital Water Samples from Eastern Turkey

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Abstract

Objective: Legionnaires' disease is a fatal form of pneumonia brought on by an infection mostly caused by the 60 different species of Legionella pneumophila. Legionnaires' illness is caused by Legionella spp.-contaminated water systems. In this work, we sought to analyze Legionella species, serogroups (SG), and contamination in the water sources of hospitals in five regions in eastern Turkey.

Methods: Between January 2017 and December 2018, a total of 1008 samples were examined, including 2 cooling towers, 62 hot water tanks, 104 cold water tanks, and 840 faucet shower heads. Samples were collected by the standard culture method L. pneumophila SG 1, it was analyzed for L. pneumophila SG 2-16 and Legionella spp. The samples were inoculated into BCYE and GVPC medium, and the colonies were assessed using a latex agglutination test, followed by species- and serotype-level identifications.

Results: In our study, a total of 1008 water samples were examined, of which 35.31% (356) belonged to 2017, while 64.68% (652) belonged to 2018. 83.33% of the water samples were taken from faucets and shower heads, 10.32% from the cold water tank, 6.15% from the hot water tank, and 0.2% from the cooling tower, and the highest positivity rate was observed in the hot water tank with 12.60%. 7.04% (71) of the samples were positive, and 16.9% (12) of the positive samples were L. pneumophila SG 1, 77.46% (55) L. pneumophila SG 2-14 was detected, while 5.63% (4) were nonpneumophila (Legionella spp) it has been determined as.

Conclusions: Legionella disease remains a significant public health threat. The water tanks of hospitals and hotels should be investigated more thoroughly, the necessary disinfection procedures should be carried out frequently. All hospitals should have water management policies, and towns and large buildings should establish comprehensive water system management programs that decrease Legionella growth and transmission. To enhance prevention measures and clinical diagnosis, we also need quicker ways of detecting Legionella in water systems and clinical samples.

Keywords: Legionella pneumophila, water samples, hospital, BCYE, GVPC

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Türkiye'nin Doğusundaki Hastanelerden Alınan Su Örneklerinde Legionella pneumophila Varlığı

Öz

Amaç: Lejyoner hastalığı, 60 türü tanımlanan, ağırlıklı olarak Legionella pneumophila' ya bağlı enfeksiyonun neden olduğu ölümcül bir pnömonidir. Legionella spp. ile kirlenmiş su sistemleri lejyoner hastalığının sorumlu kaynaklarıdır. Bu çalışmada Türkiye'nin doğusunda bulunan beş ilin hastanelerinin su kaynaklarında, Legionella kontaminasyonunu, Legionella türlerini ve sero gruplarını (SG) retrospektif olarak değerlendirmeyi amaçladık.

Yöntemler: Ocak 2017 ile Aralık 2018 arasında, 2 soğutma kulesi, 62 sıcak su tankı, 104 soğuk su deposu, 840 musluk duş başlığı örneği olmak üzere toplam 1008 örnek incelenmiştir. Örnekler, standart kültür yöntemi ile L. pneumophila SG 1, L. pneumophila SG 2-16 ve Legionella spp açısından analiz edilmiştir. Örnekler BCYE ve GVPC besiyerlerine inoküle edilmiş ve koloniler lateks aglütinasyon testi kullanılarak değerlendirilmiş, ardından tür ve serotip düzeyinde tanımlamalar yapılmıştır.

Bulgular: Çalışmamızda, toplam 1008 su örneği incelenmiş olup, bunların %35.31'i (356) 2017 yılına ait iken, %64.68'i (652) 2018 yılına aittir. Su örneklerinin %83.33 musluk ve duş başlıklarından, %10.32'si soğuk su deposundan, %6.15'i sıcak su tankından ve %0.2' si ise soğutma kulesinden alınmış olup en fazla pozitiflik oranı %12.60 ile sıcak sun tankında görülmüştür. Örneklerin %7.04'ü (71) pozitif olup, pozitif örneklerin %16.9'u (12) L. pneumophila SG 1, %77.46 'sı (55) L. pneumophila SG 2-14 olarak tespit edilirken, % 5.63'ü (4) nonpneumophila (Legionella spp.) olarak tespit edilmiştir.

Sonuçlar: Legionella hastalığı, önemli bir halk sağlığı tehdidi olmaya devam etmektedir. Hastanelerin ve otellerin su depoları daha kapsamlı araştırılmalı, gerekli dezenfeksiyon işlemleri sık sık yapılmalıdır. Bu enfeksiyonları önlemek için, belediyelerin ve büyük binaların personeli, Legionella büyümesini ve bulaşmasını azaltan etkili su sistemi yönetimi programları uygulamalı ve tüm hastanelerin su yönetimi politikaları olmalıdır. Ayrıca, önleme stratejilerini ve klinik teşhisi geliştirmek için su sistemlerinde ve klinik örneklerde Legionella'yı tespit etmek için daha hızlı yöntemlere ihtiyacımız var.

Anahtar kelimeler: Legionella pneumophila, su örnekleri, hastane, BCYE, GVPC.

INTRODUCTION

Legionnaires' disease (LD), first appeared in the United States in 1976 and was described in 1977, it is a disease with an L. pneumophila effect that can range from a mild lower respiratory tract infection to a coma, more often with pneumoniae[1]. Legionella bacteria, LD, Pontiac fever, and extrapulmonary syndrome are the causative agents. Pontiac fever is a community-acquired, travel-related, and nosocomial-related opportunistic pathogen that leads to a febrile picture with flu-like symptoms, LD lung, and extrapulmonary syndrome leading to serious clinical pictures involving extrapulmonary organs[2]. It is noted that ~70% of LD cases are of community origin, ~20% are travel-related, and ~10% are nasocomial[3]. Studies conducted show that 74-91% of patients are 50 years of age or older, and men are 1.4 to 4.3 times more common than women[4].

The prevalence of Legionnaires' disease is rising on a global scale. From 4921 in 2011 to 11.343 in 2018, the number of reported cases in Europe grew by 300%, and from 2301 in 2005 to 7104 in 2018 in the USA[5,6]. Globally, the death rate for legionnaires' disease ranges from 2.2 to 10.3%, with Singapore reporting the lowest incidence and European nations reporting the highest rate[4]. Hospital epidemics have a mortality rate that can reach 48%[7].

There are 60 species and 80 distinct serotypes of Legionella[8]. WHO estimates that only 5-10% of illnesses are brought on by other Legionella species (L. bozemanii, L. dumoffii, L. micdadei, and L. longbeachae), whereas 20–30% are brought on by other L. pneumophila serogroups. The majority of clinical cases of LD are caused by L. pneumophila (L. pneumophila 1), a member of serogroup 1 (SG)[9]. L. pneumophila accounts for around ~90% of LD cases. Legionella species may persist for a long period in aquatic environments and grow in the presence of free-living protozoa and biocides, such as chlorine[10]. More and more reports of Legionella contamination in cooling towers, spas, foot spas, and drinking water

systems of lodgings, nursing homes, and healthcare institutions are being made[11]. By inhaling or aspirating polluted aerosols or water, transmission can happen. Aerosols are solid or liquid particles suspended in air and can contain particles of any size[12]. The biofilm layers in the water and the amoeba and the cilia protozoa serve as both a food source and a shelter in adverse conditions and are survival surfaces for Legionella[13]. Studies show that 20 amoeba and two ciliated protozoa are hosts for Legionella. Although the number of studies related to water samples is limited in Turkey, colonization was detected in hotels by 10-76.2%, hospitals by 7-27.2%, houses by 21.3%, cooling towers by 26%, and hot spring thermal waters by 11%[14].

Legionnaires' disease surveillance is carried out to prevent cases and outbreaks at specified intervals in hospital-acquired Legionnaires' disease and to investigate the source in case of reported cases. Within the scope of the "Control Program" of the Ministry of Health, General Directorate of Public Health, Department of Infectious Diseases, Legionella research, prevention of the disease in all accommodation units, especially hotels, and hospitals, and the measures to be taken when necessary are carried out within the legal framework. The procedures and principles regarding the measures to be taken to be prepared against Legionnaires' disease, to prevent and combat the disease, and the procedures and principles regarding the notification of the disease are implemented and in this context, the regulation published on "Legionnaires' Disease Control Procedures and Principles" is complied with. "Legionnaires' Disease Control Program Guide" has been developed to ensure standardization of the work to be carried out within the scope of the regulation. In the light of the regulations made with this guide; it is aimed to ensure that Legionnaires' Disease is diagnosed within the framework of certain standards, to obtain accurate notifications, to organize the work to be carried out in accommodation units within the scope of environmental surveillance, and to reveal the extent of the disease in Turkey. In addition, Legionella samples are usually taken every 3 months by the Environmental Health Unit of Public Health or Community Health Centers and analyzed by the relevant Public Health Laboratory[1].

In this study, we aimed to retrospectively evaluate the Legionella contamination in the water systems of hospitals belonging to 5 eastern cities of our country, related Legionella species, and serogroups.

METHODS

Between January 2017 and December 2018, a total of 1008 water samples taken from various water systems of hospitals located in the provinces of Van, Bitlis, Muş, Igdır, and Hakkâri were examined for Legionella (Table I). In our study, samples were taken from a total of 35 hospitals, 13 from Van, 6 from Hakkâri, 7 from Bitlis, 7 from Muş, and 2 from Iğdır.

Table I: Culture and serogrouping results of samples collected from water systems

Sample type	Samples n (%)	*Legionella* reproduce n (%)	Lateks aglütinasyon serogrup results	n
Faucet Shower Head	840 (83.33)	62 (7.38)	*L. pneumophila* SG 1	9
			L. pneumophila SG 2-16	49
			Legionella spp.	4
Cold Water Tank	104 (10.32)	1 (0.98)	*L. pneumophila* SG 1	1
Hot Water Tank	62 (6.15)	8 (12.60)	*L. pneumophila* SG 1	2
			L. pneumophila SG 2-16	6
Cooling Tower	2 (0.2)	0		0
Total	**1008**	**71**		**71(7.04%)**

Water samples (100 ml) were taken in sterile, sealed, and twist-capped bottles. Buffered Charcoal Extract Agar (BCYE) and Glycine Vancomycin Polymyxin Cycloheximide (GVPC) media were used for Legionella isolation[15].

Taking into account the characteristics of the sampling point, water was sown "directly" and/or "after acid treatment" and/or "after filtering and acid treatment" on the culture plates. Water samples taken from the tank, fountain, faucet, and shower heads were divided into two growth media, using a drigalsik spatula to spread one with 0.1 ml BCYE and the other one with 0,1 ml GVPC. 50 ml of water was taken from the same sample and passed through a 0.2 µm membrane filter. The resulting filter was placed in 5 ml of sterile water and vortexed for 30 sec. After adding 2 ml of the 5 ml solution in which the filter was located to the 2 ml HCl-KCl (pH 2.2) acid solution prepared in advance, 3 minutes have waited and 0.1 ml BCYE and GVPC media were taken and cultivation was performed by spreading with a drigalski spatula. Two to six plaque media were used for each water sample (Figure 1).

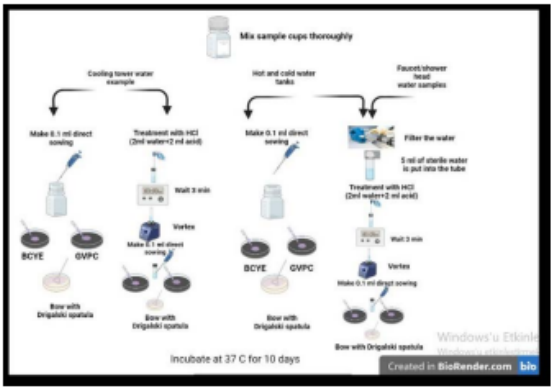

Figure 1: Flow chart for the preparation of samples taken from different sampling points for culture in the laboratory and their cultivation in primary culture media.

The media that were spread were placed in an incubator with a temperature of 36-37 °C and humidity of at least 85% and left for incubation. First of all, starting on day 3, assessments were started and followed by days 5, 7, and 10. Colonies that are considered suspicious were assessed after the 3rd day, the colony was evaluated using a microscope and UV light; when examined with the naked eye, its surfaces are smooth, slightly dished, gray-white, 1-3 mm in diameter; under a microscope, colonies with pink, purple, green or frosted glass edges are evaluated as the target colony (HSGM 2014). A single colony passage was made from suspected Legionella colonies to the BCYE and blood agar medium (Figure 2).

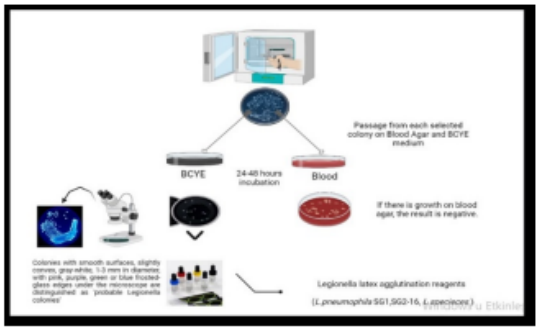

Figure 2: Parallel passage method and subsequent identification steps from suspicious colonies determined by colony microscope in primary culture of water sample to blood agar and BCYE agar plates.

The passages left for incubation were evaluated a day later, but if there was no reproduction, they were incubated for another day. Colonies that did reproduce in BCYE media and did not reproduce in blood agar were evaluated as suspected Legionella. Colonies that were considered to be Legionella were typed by performing a latex agglutination test (Migrogen M45, UK) for serological examination.

This study was approved by the local institutional ethical board (University Kafkas-Interventional Clinical Research Ethics Committee; File Number: 2018/11).

RESULTS

Between January 2017- December 2018, a total of 1008 water samples belonging to various water systems of hospitals located in the eastern provinces of Turkey were bacteriologically and serologically investigated for Legionella bacteria in our study. Of these, 35.31% (356) belong to the year 2017, while 64.68% (652) belong to the year 2018. When

the seasonal distribution of the samples was examined, 25.49% (257) of them were taken in autumn, 5.65% (57) in winter, 25.59% (258) in spring, and 46.23% (466) in summer. The positivity rate in the autumn, winter, spring, and summer seasons are: 29.57 % (21), 7.04 % (5), 33.8% (24), 29.57% (21), and the greatest positivity was observed in the spring months (Graphic 1).

Graphic 1. The distribution rate of Legionella samples according to seasons.

As a result of the study, the results of the latex agglutination test and serogroup (SG) from Legionella colonies are shown in Table II. 7.04% (71) of the samples were positive and 16.9% (12) of the positive samples were L. pneumophila SG 1, 77.46% (55) of them were L. pneumophila SG 2-14, while 5.63% (4) were nonpneumophila.

Table II: Serogrouping results of samples collected from water systems according to cities and years

City	Year	Negative n (%)	Positive n (%)	Latex agglutination serogrup results	n
Van	2017	232	8	*L. pneumophila* SG 1	4
				L. pneumophila SG 2-16	2
				Legionella spp.	2
	2018	292	15	*L. pneumophila* SG 1	8
				L. pneumophila SG 2-16	7
				Legionella spp.	0
Bitlis	2017	31	16	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	15
				Legionella spp.	1
	2018	167	23	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	22
				Legionella spp.	1
Muş	2017	67	2	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	2
				Legionella spp.	0
	2018	74	4	*L. pneumophila* SG 1	2
				L. pneumophila SG 2-16	4
				Legionella spp.	0
Hakkâri	2017	0	0	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	0
				Legionella spp.	0
	2018	33	0	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	0
				Legionella spp.	0
Iğdır	2017	0	0	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	0
				Legionella spp.	0
	2018	41	3	*L. pneumophila* SG 1	0
				L. pneumophila SG 2-16	3
				Legionella spp.	0
Total		**937**	**71**		**71 (%)**

DISCUSSION

Bacteria of the genus Legionella, which maintain their viability by colonizing water systems at different temperatures, cause Legionnaires' disease outbreaks, but also reduce the quality of life and cause economic losses. In addition, these bacteria are not affected much by adverse conditions, as they can live as intracellular parasites in unicellular creatures that can be found in waters with biofilm layers formed in water sources. The presence of bacteria in public living spaces such as hospitals poses a risk of infection, especially in patients with immune system problems. In addition, it can maintain its vitality for two hours in cooling systems such as air conditioning, it can easily multiply in water pipes and spread to the environment.

Although water-borne diseases are significantly reduced by water management and sanitation, outbreaks continue to occur. The US Centers for Disease Control and Prevention (CDC) blamed Legionella for 66% of the outbreaks and 6% of the diseases that occurred between 2011 and 2012. It has been determined that 66% of Legionella outbreak was caused by building plumbing and 13% was caused by untreated groundwater[15].

When the studies conducted in our country were examined; In their study on 43 samples belonging to Aktaş's environmental and hospital water systems, they isolated 69.76% of Legionella. Of these, 26.66% were isolated from shower heads, 30% from faucets, and 43.33% from samples they took from warehouses. In addition, there are 8 Legionella spp from the warehouses they have isolated. They isolated bacteria from samples taken from the largest number of warehouses[16]. In Ayhan's thesis study conducted on 215 samples, they detected Legionella in 13.74% of them, reported that 26.43% were from warehouses and 16.85% were from faucet shower heads[17]. In a different investigation with 2.025 samples, 3.2% of the water samples from hospitals (94.8%), hotels (1.6%), Turkish baths (0.2%), and shopping malls (0.2%) had Legionella. Of the 65 samples that tested positive, L. pneumophila serogroup 2-14 was discovered in 46 (70.8%) of them[18]. Mouchtori and co isolated Legionella in 20.8% (Mouchtouri et al. 2007) of 385 hot and cold water samples belonging to hotels in Greece, Borella and co isolated Legionella in 22.6% of shower heads and tap water in Italy, Zietz and co isolated Legionella in 26% of hot water samples in Germany[19,20]. In our study, 1008 water samples were examined and Legionella was isolated from 7.04% of them. 83.33% of the samples were taken from faucets and shower heads, 10.32% from the cold water tank, 6.15% from the hot water tank, and 0.2% from the cooling tower, and the highest positivity rate was observed in the hot water tank with 12.60%. This situation has shown similarities with the literature and has been interpreted as an advantage for the colonization of these organisms by warm and stagnant waters.

Of the 8 Legionella isolates obtained from a water sample of 150 units made in our country, 6 were found to have L. pneumophila SG 2-15, and 2 of them have Legionella spp.[21]. Again, in another study conducted in schools, hospitals, and hotels, they detected Legionella in 29.71% of 313 samples, 79.56% of them were L. pneumophila SG 2-14, and 20.43% were L. pneumophila SG 1[22]. In another study, Legionella has detected in 13.74% of the samples taken, and 61.11% were L. pneumophila SG 2-15, Legionella spp in 27.77%, and L. pneumophila SG 1 at 11.11%[23]. When the studies conducted are examined worldwide; Laganà and co, detected Legionella in 64% of 92 water samples, 36% of which were L. pneumophila SG 1, 51%, L. pneumophila SG 2-14, and 13% detected both serogroups[24]. According to Goutziana and co a total of 96 Legionella isolates were detected in the study of 90.6% and 44.8% of L. pneumophila SG 1 account for 44.8% of it was noted L. pneumophila SG 2-14 and the remaining 9.4% were also detected as Legionella spp.[25]. In our study, 7.04% of the samples were positive and 16.9% of the positive samples were L. pneumophila SG 1,77.46% of L. pneumophila SG 2-14, 5.63% Legionella spp. it has been determined that the most we have detected L.

pneumophila SG 2-14 and our results are consistent with the literature.

When a literature review was conducted, there was no similar study in which a study was conducted by month. In our study, the highest positivity rate was observed in the spring months (33.8%) and then in the summer and autumn at the same rate. We attribute this to the fact that the spring seasons are a transitional period and it is the period when the heaters do not burn, so they start to heat up with air conditioners. In the summer season, we think that it is due to the fact that it is preferred to cool down with the air conditioning in the extreme temperatures.

CONCLUSIONS

Legionnaires' disease is considered a preventable infection, especially hospital-acquired LD, due to its potential to cause an epidemic, and is a disease of public health importance and is monitored under the control program. Legionella infections are not well known in our country and are not considered by clinicians in the first place. To be examined for Legionella, especially in cases of nosocomial pneumonia, it should be passed to the system as a routine test in the microbiology laboratories of all hospitals. In Legionella analyses performed by Health Directorates at specific intervals with water samples taken from hospitals, routine use of urinary antigen test should be mandatory in nosocomial pneumonias encountered in the hospital in case of pneumophila SG 1 reproduction.

To prevent the disease, it is also recommended that hospitals conduct risk assessments, implement routine preventive measures, conduct a program with active case surveillance, and periodically clean and decontaminate these areas at frequent intervals.

Legionella culture analysis results from a minimum of 5 and maximum of 12 days, which would cause it to fail in managing an outbreak of the epidemic that results in more comprehensive epidemiological research in laboratories for PCR, DFA, PCR, and FISH and especially that we believe should be done routinely supported by molecular techniques. In the studies, seasonal differentiation of samples, evaluation of the sampled waters in terms of temperature, pH, and chlorine value; it is proposed to evaluate the sampled structures and the water system in terms of parameters such as the year of use, material, and the way the water is heated.

Ethics Committee Approval: This study was approved by the local institutional ethics committee (Van Training and Research Hospital Clinical Research Ethics Committee; File no: 2018/11).

Conflict of Interest: The authors declared no conflicts of interest.

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Anexo 5. Capturas del artículo n°5 titulado: Evaluation of Legionella pneumophila: Decrease in Hot Water Network of Four Hospital Buildings after Installation of Electron Time Flow Taps.

water

MDPI

Article

Evaluation of *Legionella pneumophila* Decrease in Hot Water Network of Four Hospital Buildings after Installation of Electron Time Flow Taps

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Abstract: *Legionella* spp. control is a critical issue in hospital with old water networks. Chemical disinfection methods are applied as a control measure over prolonged time periods, but *Legionella* may be resistant to chemical agents in pipeworks with low flow and frequent water stagnation. We evaluated *Legionella* spp. colonization in the hot water network of Italian hospitals after the installation of time flow taps (TFTs). In the period between 2017 and 2019, TFTs were installed in four hospital water networks. They were programmed in order to obtain a hot water flow of 192 L/day from each TFTs. A continuous chlorination system (chlorine dioxide) and a cold water pre-filtration device were applied in all the buildings. Before and after TFT installation, *Legionella* spp. was investigated at scheduled times. Before TFT installation, *Legionella pneumophila* was detected in all the hospitals with counts ranging from 2×10^2 to 1.4×10^5 CFU/L. After TFT installation, a loss in *Legionella pneumophila* culturability was always achieved in the period between 24 h and 15 days. Total chlorine concentration (Cl_2) was detected in the range between 0.23 and 0.36 mg/L while temperature values were from 44.8 to 53.2 °C. TFTs together with chemical disinfection represent a method which improve water quality and disinfectant efficacy, reducing *Legionella* colonization in dead-end sections.

Keywords: *Legionella*; electron time flow taps; hospital water network; chlorine dioxide

1. Introduction

Legionella bacteria are environmental organisms found in water, soil, and other environments. Typically, they are transmitted to humans via the inhalation of contaminated aerosols and they may cause fulminant pneumonia or mild Pontiac fever. In particular, nosocomial *Legionella* spp. infection may affect immunosuppressed, transplant patients, and people undergoing aggressive chemotherapy [1,2].

According to the European Center for Disease Prevention and Control (ECDC), the rate of Legionnaires' disease in Europe in 2017 was 18 cases per million inhabitants. During the same period, in Europe, 9 out of the 30 countries reported 28 legionellosis outbreaks, either community or hospital acquired. A 30% increase in the number of reported cases in Europe was observed in 2017 compared with 2016, with an increase in the overall European notification rate from 1.4 to 1.8 per 100,000 population [3,4].

The latest Italian data, related to 2017, give 2014 cases of Legionnaires' disease in the Italian population, corresponding to a notification rate of 33.2 cases per million inhabitants. It must be noted that 2017 has seen an increase of 26% in the cases when compared with the previous three years. Of the 2014 notified cases, 124 (6.2%) were hospital acquired while 1580 (78.5%) were community-acquired infections [5].

International and Italian guidelines for Legionnaires' disease control suggest that large hospital water networks and high-volume hot water storage tanks provide optimal conditions for *Legionella* colonization; the complexity of the water distribution systems and the presence of old and corroded pipelines and dead-end branches may promote the growth of *Legionella* in hospital water networks [6,7]. These critical points in water distribution systems cause a low water flow with a higher likelihood of biofilm development. Once microorganisms enter a system, they may be incorporated into biofilm, which is a primary cause of scarce water quality. Biofilm provides a nutrient source and protection from unfavorable environmental conditions. Several variables impact biofilm formation, including fluctuating water usage and transient stagnation [8–11]. In a previous study, we reported the experience of an Italian hospital where a cost-effectiveness strategy for *Legionella* control in the hot water distribution system was applied with continuous disinfection treatment and the installation of time flow taps (TFTs) in correspondence of all the dead-end branches, with the aim to increase the water flow of 196 L/day. *Legionella pneumophila* sg2–14 was initially detected in all the sampling points despite the presence of chemical disinfection. After eight months of TFTs activity, *Legionella* spp. was not detected anymore [12].

Based on that preliminary experience, the aim of this study is the application of the same method in teaching hospitals hosting high-risk patients. In this manuscript, we report the data related to the decrease in *Legionella* spp. contamination in the hot water networks of four hospitals located in Northwestern Tuscany (Italy) from 2017 to 2019.

2. Materials and Methods

2.1. Settings

The healthcare setting, called Hospital 1, is a 60-bed Internal and Emergency Medicine Hospital. This structure has been active since 1950. The hospital architectural structure is a monoblock with a central plate on four levels. Hospital 2 (Oncology Unit) is a monoblock with a central plate with three levels which has been active since 1972. It has 30 beds. Hospital 3 is a 320-bed General and Transplant Surgery Unit which has been active since 2007. It is organized in five blocks with four levels. Hospital 4 is a 11-bed Senology Unit which has been active since 1987. It has a central plate on four levels.

2.2. Water Disinfection

Within the Water Safety Plan (WSP) implementation program, hot water system treatment and a systematic monitoring program with sampling at points of use began in all hospitals.

The hospital WSP is a self-control manual aimed to manage the microbiological water risk from waterborne pathogens, such as *Legionella* spp. This protocol is a technical guide which describes the control critical points of the water systems (dead-end branches, pipework materials, devices, etc.), the water samplings for microbiological tests, the disinfection procedure of water networks, and all responsibilities related to plant management.

Municipal drinking water was pre-filtered before entering the hospital hot water distribution system. Despite the old and galvanized steel-made pipelines, the hot water network was treated with continuous chlorine dioxide disinfection.

Continuous disinfection with chlorine dioxide was applied in water networks. This method was used one day before TFT installation.

In hospital water stations, a chemical feed pump, chlorine solution tank, and chlorine residual monitoring kit were installed.

Hot water was recirculated in order to obtain an energy saving. After the identification of all the dead-end branches present in the hot water distribution system, TFTs were installed corresponding to the most critical points.

They present an adjustable flowmeter in order to measure the scheduled water flow. TFTs were programmed in order to obtain a hot water flushing of 1 minute every 2 h (192 liters per day from each TFT), as described elsewhere [12].

The TFTs, continuous chlorine dioxide disinfection, and municipal water pre-filtration plant were simultaneously installed in Hospital 1, Hospital 2, and Hospital 4. Four TFTs were placed in these buildings.

In Hospital 3, the continuous chlorination method was already present before TFT installation. Seven TFTs were simultaneously installed.

2.3. *Hot Water Samplings and* Legionella *spp. Detection*

According to all the hospital WSP programs, the hot water distribution system was sampled at the recirculation point of the central heating system (R), at the exit of the hot water production boiler (B), at each TFT, and at two points of use without TFTs (P1 and P2).

TFTs were installed close to some dead-end branches of the water networks (storage rooms, kitchens, and lockers rooms), while P1 and P2 were chosen from medical stays or surgery areas.

Legionella spp. detection and physical–chemical parameter assessment (total chlorine concentration, pH, and water temperature) were performed according to the following schedule:

- One week before TFT installation (Time 0 or T0);
- The day after TFT installation (Time 1 or T1);
- Seven days after TFT installation (Time 2 or T2);
- Fifteen days after TFT installation (Time 3 or T3);
- One month after TFT installation, and then regularly on a monthly basis (Time 4 or T4, Time 5 or T5, etc.).

All physical–chemical parameter were measured in triplicate before and after TFT installation.

Samplings were performed after five minutes of water flushing. Hot water was collected in sterile bottles having sodium thiosulfate (20 mg/L). Temperature and total chlorine values were detected after the flushing.

Total chlorine concentration was determined by the colorimetric Visocolor HE® test (Macherey-Nagel, Düren, Germany).

Isolation of *Legionella* spp. in hot water samples was performed as suggested by the international standards procedure ISO11731 [13]. Briefly, one liter of water was filtrated through a 0.2 µm diameter membrane (Millipore, Billerica, MA, USA); the membrane was then immersed in 10 mL of the same water and sonicated for 5 min, allowing the detachment of the cells. The suspension was thermally inactivated by incubation at 50 °C for 30 min. Afterwards, 0.1 mL of the suspension was plated on *Legionella* BMPA selective medium (Oxoid Ltd., Basingstoke, Hampshire, UK) and the plates were placed in one incubator at 37 °C for 7–10 days within jars with a modified atmosphere (2.5% CO_2) Finally, 10% of the *Legionella*-suspected colonies that grew on the medium were subjected to confirmation test using BCYE medium and BCYE without cysteine medium (Oxoid Ltd., Basingstoke, Hampshire, UK). Confirmed colonies were subjected to species and serogroup identification analysis using a multipurpose latex agglutination test (*Legionella* Latex Test, Oxoid Ltd., Basingstoke, Hampshire, UK).

Chemical samplings were taken at the same points chosen for microbiology tests. Chemical analyses were performed one week before TFT installation, six months after TFT installation, and then regularly on a six-monthly basis. Chemical parameters such as iron ions, zinc ions, and trihalomethanes (THMs) were determined as established by Council Directive 98/83/EC [14].

2.4. Statistical Analysis

The Kolmogorov–Smirnov test was performed to verify normality of distributions. For each hospital, Kruskal–Wallis test and Dunn's test were used to compare *Legionella* spp. counts detected before and after TFT installations, as described elsewhere [12]. Power tests were carried out to estimate the sample sizes. The 1-beta values of the significant variables were >0.8, demonstrating that sample sizes were acceptable. The statistical analysis was fulfilled using IBM SPSS software package version 17.0.1.

3. Results

3.1. Legionella spp.

In *Hospital 1*, before the TFT installation, high *Legionella pneumophila* sg2–16 concentrations were detected in all the tested water point of use. Concentrations ranging from 2×10^2 to 2.6×10^4 CFU/L, with a mean value of $9.2 \times 10^3 \pm 1.1 \times 10^3$ CFU/L, were observed in the hot water samples. Following the implementation of TFTs and water treatment, from September 2017 onwards, we detected *Legionella pneumophila* sg2–16 for at least seven days, with concentrations ranging from 1×10^2 to 8.8×10^4 CFU/L and a mean count of $3.2 \times 10^4 \pm 2.9 \times 10^4$ CFU/L. A significant decrease in *Legionella pneumophila* sg2–16 contamination was obtained at the fifteenth and the thirtieth day (T3 and T4). After two months, *Legionella* spp. was no longer recovered (Figure 1).

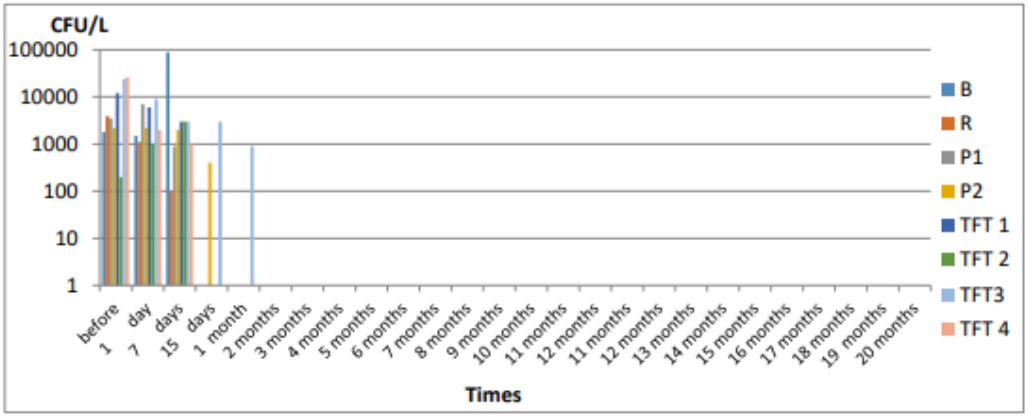

Figure 1. *Legionella pneumophila* sg2–16 counts at all the sampling points during the whole period of the study in Hospital 1. B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4.

High *Legionella pneumophila* sg1 concentrations were detected in *Hospital 2* before TFT installation (from 3×10^2 to 1.5×10^3 CFU/L, mean of $8.8 \times 10^2 \pm 1.5 \times 10^2$ CFU/L). From April 2018, *Legionella pneumophila* sg1 was detected for at least four days, with a mean concentration of 1.8×10^2 CFU/L. *Legionella* spp. could not be detected after seven days (T2), (Figure 2).

In Hospital 3, despite the presence of the continuous chemical chlorination, *Legionella pneumophila* sg1 was detected in all the hot water samples at T0. *Legionella* concentrations ranged from 2.1×10^4 to 1.4×10^5 CFU/L (mean of $5.5 \times 10^4 \pm 3.1 \times 10^3$ CFU/L). After TFT installation, from November 2018, *Legionella pneumophila* sg1 was still present for at least two weeks (from 1×10^2 to 2.4×10^3 CFU/L). Absence of *Legionella pneumophila* sg1 was obtained from the fifteenth day (T3) (Figure 3).

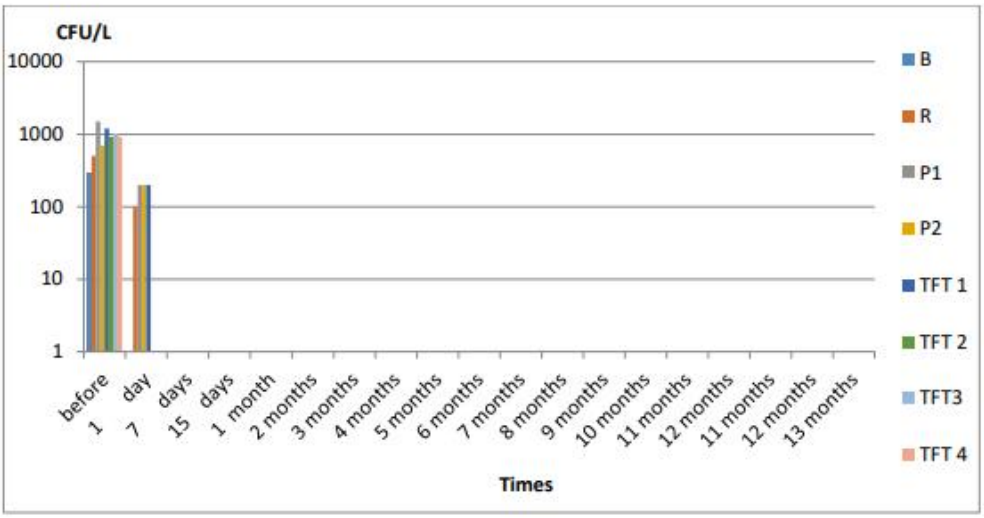

Figure 2. *Legionella pneumophila* sg1 counts at all the sampling points during the whole period of the study in Hospital 2. (B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4).

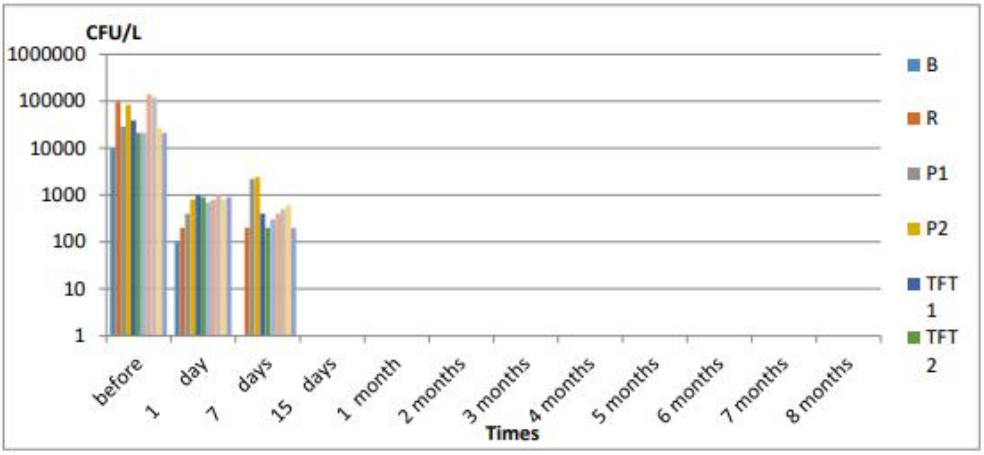

Figure 3. *Legionella pneumophila* sg1 counts at all the sampling points during the whole period of the study in Hospital 3. B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4; TFT5 = time flow tap 5; TFT6 = time flow tap 6; TFT7 = time flow tap 7.

Before the TFT installation, *Legionella pneumophila* sg1 was detected in *Hospital 4*. *Legionella* counts ranged from 1×10^2 to 1.9×10^4 CFU/L, with a mean value of $9.9 \times 10^3 \pm 2.8 \times 10^3$ CFU/L. Following the implementation of TFTs and water treatment, from February 2018, *Legionella pneumophila* sg1 was detected for one day (2.2×10^2 CFU/L). *Legionella pneumophila* sg1 was no longer detected from the seventh day (T2) (Figure 4).

In all hospitals, statistically significant reductions of *Legionella* counts were detected from the period were TFTs were absent to the period when the water flushing was increased ($p < 0.001$).

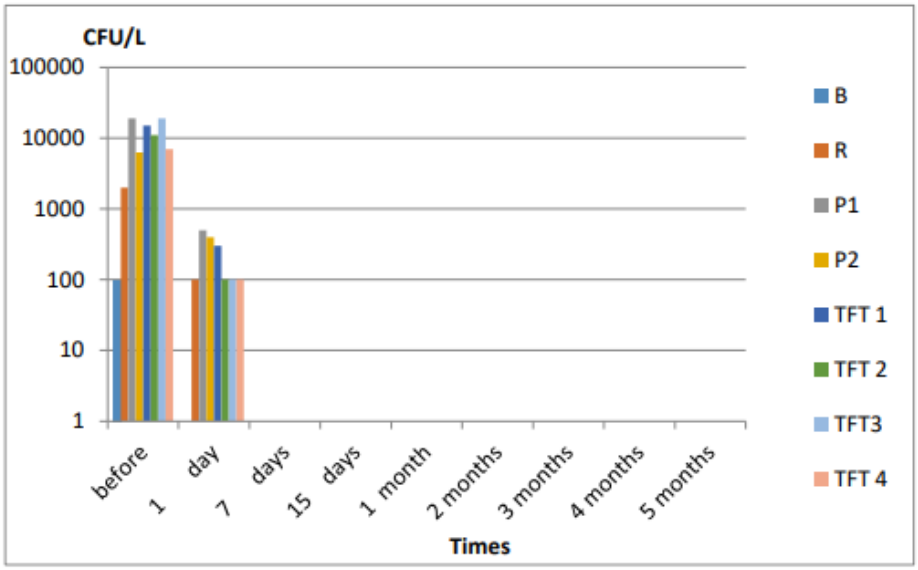

Figure 4. *Legionella pneumophila* sg1 counts at all the sampling points during the whole period of the study in Hospital 4. B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4.

3.2. Physical–Chemical Parameters

In all hospitals, good chemical quality was observed in all hot water samples, and all values were within the limits recommended by Council Directive 98/83/EC [14].

In Hospital 1, the hot water temperature ranged from 36.4 ± 1.9 to 48.4 ± 2.8 °C before TFT installation. From the first day, mean temperature values ranged from 45.5 ± 3.2 and 53.2 ± 1.6 °C while continuous chlorination was maintained at mean values between 0.23 ± 0.04 to 0.31 ± 0.06 mg/L (Table 1).

Before TFT installation, the hot water temperature ranged from 35.8 ± 3.9 and 43.5 ± 8.4 °C in Hospital 2.

After TFT installation, the mean temperature values ranged from 46.6 ± 2.1 and 51.4 ± 1.7 °C, while the continuous chlorination was maintained at mean values between 0.23 ± 0.06 to 0.35 ± 0.04 mg/L (Table 2).

Table 1. Mean values of physical–chemical and chemical parameters assessed at each sampling points before and after TFT installation in Hospital 1. B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4.

BEFORE TFT INSTALLATION (September 2017)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0	48.4 ± 2.8	6.52 ± 0.31	52 ± 4.5	67 ± 2.6	<3
R	0	38.8 ± 1.9	6.50 ± 0.12	69 ± 4.3	73 ± 1.9	<3
P1	0	38.8 ± 2.1	6.60 ± 0.16	57 ± 3.9	82 ± 3.8	<3
P2	0	36.4 ± 1.9	6.57 ± 0.11	98 ± 2.6	79 ± 6.2	<3
TFT 1	0	39.5 ± 1.6	6.58 ± 0.09	74 ± 5.9	65 ± 7.1	<3
TFT 2	0	37.7 ± 1.7	6.70 ± 0.31	61 ± 6.6	81 ± 6.0	<3
TFT 3	0	38.1 ± 2.5	6.60 ± 0.09	69 ± 5.7	83 ± 5.7	<3
TFT 4	0	37.6 ± 2.2	6.52 ± 0.06	70 ± 4.2	77 ± 6.4	<3
AFTER TFT INSTALLATION (September 2017–July 2019)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0.31 ± 0.06	53.2 ± 1.6	6.59 ± 0.18	59 ± 3.1	74 ± 3	<3
R	0.23 ± 0.04	49.6 ± 2.1	6.60 ± 0.11	77 ± 3.6	81 ± 4.1	<3
P1	0.29 ± 0.09	47.4 ± 1.2	6.56 ± 0.09	63 ± 2.9	79 ± 3.3	<3
P2	0.27 ± 0.09	45.5 ± 3.2	6.5 ± 0.08	77 ± 4.2	66 ± 4.2	<3
TFT 1	0.26 ± 0.08	48.6 ± 2.5	6.58 ± 0.13	68 ± 3.8	78 ± 2.6	<3
TFT 2	0.24 ± 0.04	49.8 ± 1.5	6.51 ± 0.10	71 ± 4.6	82 ± 5.2	<3
TFT 3	0.26 ± 0.05	48.3 ± 2.1	6.61 ± 0.07	74 ± 2.3	84 ± 3.9	<3
TFT 4	0.26 ± 0.08	47.3 ± 2.2	6.58 ± 0.09	75 ± 3.3	79 ± 3.6	<3

Table 2. Mean values of physical–chemical and chemical parameters assessed at each sampling points before and after TFT installation in Hospital 2. B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4.

BEFORE TFT INSTALLATION (April 2018)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0	43.5 ± 8.4	6.72 ± 0.18	66 ± 2.2	64 ± 1.1	<3
R	0	37.6 ± 9.2	6.70 ± 0.19	68 ± 2.1	66 ± 1.6	<3
P1	0	39.2 ± 5.4	6.74 ± 0.21	61 ± 1.9	64 ± 1.0	<3
P2	0	35.8 ± 3.9	6.80 ± 0.20	78 ± 1.1	80 ± 0.9	<3
TFT 1	0	38.8 ± 4.8	6.78 ± 0.16	79 ± 1.8	85 ± 0.7	<3
TFT 2	0	39.4 ± 5.9	6.78 ± 0.19	65 ± 2.0	87 ± 1.2	<3
TFT 3	0	39.1 ± 4.6	6.76 ± 0.16	69 ± 2.2	86 ± 1.3	<3
TFT 4	0	36.9 ± 6.9	6.80 ± 0.25	73 ± 2.1	79 ± 1.0	<3
AFTER TFT INSTALLATION (April 2018–July 2019)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0.35 ± 0.07	51.4 ± 1.7	6.79 ± 0.11	74 ± 0.19	63 ± 0.19	<3
R	0.26 ± 0.06	47.6 ± 1.9	6.70 ± 0.10	69 ± 0.15	68 ± 0.13	<3
P1	0.24 ± 0.09	47.6 ± 1.4	6.60 ± 0.10	63 ± 0.15	66 ± 0.14	<3
P2	0.23 ± 0.08	46.6 ± 2.1	6.62 ± 0.09	75 ± 0.11	81 ± 0.18	<3
TFT 1	0.25 ± 0.06	47.5 ± 2.1	6.68 ± 0.11	79 ± 0.17	84 ± 0.17	<3
TFT 2	0.27 ± 0.05	47.9 ± 1.6	6.61 ± 0.14	70 ± 0.12	82 ± 0.12	<3
TFT 3	0.26 ± 0.03	48.4 ± 1.7	6.64 ± 0.11	74 ± 0.17	85 ± 0.19	<3
TFT 4	0.27 ± 0.05	46.9 ± 1.8	6.78 ± 0.11	76 ± 0.18	81 ± 0.11	<3

In Hospital 3, total chlorine concentrations were between 0 to 0.1 ± 0.02 mg/L, while hot water temperature values ranged from 32.8 ± 1.8 to 50.7 ± 1.6 °C before TFT installation. Continuous chlorination was maintained at mean values between 0.25 ± 0.01 to 0.40 ± 0.01 mg/L, while mean temperature values ranged from 44.8 ± 1.8 and 50.7 ± 1.6 °C (Table 3).

In Hospital 4 the water temperature values ranged from 32.4 ± 1.8 to 39.9 ± 3.2 °C at T0.

From the first day the mean temperature values ranged from 46.4 ± 2.2 to 50.1 ± 1.9 °C and the continuous chlorination was maintained at mean values between 0.28 ± 0.06 and 0.36 ± 0.04 mg/L (Table 4).

Table 3. Mean values of physical–chemical and chemical parameters assessed at each sampling points before and after TFT installation in Hospital 3. (B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4; TFT5 = time flow tap 5; TFT6 = time flow tap 6; TFT7 = time flow tap 7).

BEFORE TFT INSTALLATION (November 2018)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0.10 ± 0.02	40.6 ± 3.1	6.80 ± 0.21	55 ± 0.8	54 ± 1.6	<3
R	0.05 ± 0.01	35.8 ± 2.5	6.78 ± 0.20	52 ± 0.9	61 ± 2.1	<3
P1	0.1 ± 0.05	40.1 ± 1.8	6.76 ± 0.15	51 ± 1.1	53 ± 2.2	<3
P2	0.0 ± 0.0	42.6 ± 1.6	6.78 ± 0.17	60 ± 0.8	54 ± 2.5	<3
TFT 1	0.05 ± 0.01	41.1 ± 1.5	6.78 ± 0.17	61 ± 0.9	61 ± 2.6	<3
TFT 2	0.05 ± 0.01	41.9 ± 2.2	6.75 ± 0.15	56 ± 1.2	58 ± 3.6	<3
TFT 3	0.05 ± 0.01	42.6 ± 3.6	6.82 ± 0.20	55 ± 1.9	57 ± 3.4	<3
TFT 4	0.05 ± 0.01	39.8 ± 3.2	6.82 ± 0.19	56 ± 2.2	55 ± 5.0	<3
TFT 5	0.05 ± 0.02	39.5 ± 3.0	6.79 ± 0.12	55 ± 2.6	60 ± 2.9	<3
TFT 6	0.05 ± 0.03	39.1 ± 2.9	6.80 ± 0.16	54 ± 2.0	59 ± 2.3	<3
TFT 7	0.05 ± 0.01	32.8 ± 1.8	6.80 ± 0.09	55 ± 1.8	56 ± 3.3	<3
AFTER TFT INSTALLATION (November 2018–July 2019)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0.40 ± 0.01	50.7 ± 1.6	6.79 ± 0.11	59 ± 0.12	55 ± 0.12	<3
R	0.25 ± 0.10	44.8 ± 1.8	6.70 ± 0.10	59 ± 0.16	57 ± 0.12	<3
P1	0.31 ± 0.10	46.5 ± 1.8	6.60 ± 0.10	61 ± 0.18	55 ± 0.11	<3
P2	0.33 ± 0.11	49.8 ± 2.2	6.62 ± 0.09	65 ± 0.13	63 ± 0.19	<3
TFT 1	0.28 ± 0.12	48.8 ± 2.0	6.68 ± 0.11	61 ± 0.11	62 ± 0.15	<3
TFT 2	0.30 ± 0.06	49.1 ± 1.8	6.61 ± 0.14	62 ± 0.10	68 ± 0.14	<3
TFT 3	0.29 ± 0.05	48.8 ± 1.6	6.64 ± 0.11	63 ± 0.11	74 ± 0.10	<3
TFT 4	0.28 ± 0.03	47.6 ± 1.6	6.78 ± 0.11	66 ± 0.13	61 ± 0.09	<3
TFT 5	0.32 ± 0.04	48.6 ± 1.2	6.80 ± 0.10	61 ± 0.09	69 ± 0.11	<3
TFT 6	0.29 ± 0.06	47.6 ± 1.4	6.69 ± 0.10	59 ± 0.13	64 ± 0.11	<3
TFT 7	0.30 ± 0.02	47.2 ± 1.3	6.72 ± 0.11	59 ± 0.12	66 ± 0.08	<3

Table 4. Mean values of physical–chemical and chemical parameters assessed at each sampling points before and after TFT installation in Hospital 4. (B = boiler; R = recirculation; P1 = point 1; P2 = point 2; TFT1 = time flow tap 1; TFT2 = time flow tap 2; TFT3 = time flow tap 3; TFT4 = time flow tap 4).

BEFORE TFT INSTALLATION (February 2019)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0	39.9 ± 3.2	6.81 ± 0.10	77 ± 0.50	69 ± 0.18	<3
R	0	37.1 ± 2.5	6.78 ± 0.12	81 ± 0.60	68 ± 0.20	<3
P1	0	35.4 ± 2.9	6.76 ± 0.13	76 ± 0.62	64 ± 0.16	<3
P2	0	34.6 ± 2.6	6.80 ± 0.09	74 ± 0.41	78 ± 0.17	<3
TFT 1	0	33.2 ± 1.8	6.79 ± 0.18	75 ± 0.52	74 ± 0.24	<3
TFT 2	0	32.4 ± 4.2	6.78 ± 0.20	71 ± 0.19	79 ± 0.20	<3
TFT 3	0	33.3 ± 3.6	6.77 ± 0.15	79 ± 0.20	75 ± 0.19	<3
TFT 4	0	34.5 ± 2.7	6.74 ± 0.11	81 ± 0.16	76 ± 0.11	<3
AFTER TFT INSTALLATION (February 2019–July 2019)						
SAMPLING POINT	**TOTAL CHLORINE (Mean ± SD mg/L)**	**TEMPERATURE (Mean ± SD °C)**	**pH (Mean ± SD)**	**IRON IONS (Mean ± SD µg/L)**	**ZINC IONS (Mean ± SD µg/L)**	**THMs (µg/L)**
B	0.36 ± 0.04	50.1 ± 1.9	6.83 ± 0.08	80 ± 0.14	73 ± 0.17	<3
R	0.30 ± 0.04	46.4 ± 2.2	6.79 ± 0.08	83 ± 0.12	71 ± 0.15	<3
P1	0.34 ± 0.07	48.5 ± 1.3	6.79 ± 0.09	79 ± 0.09	72 ± 0.18	<3
P2	0.29 ± 0.09	49.9 ± 2.0	6.78 ± 0.09	76 ± 0.10	80 ± 0.19	<3
TFT 1	0.31 ± 0.07	48.9 ± 2.1	6.81 ± 0.06	79 ± 0.15	80 ± 0.19	<3
TFT 2	0.32 ± 0.04	48.2 ± 1.9	6.70 ± 0.08	76 ± 0.14	79 ± 0.18	<3
TFT 3	0.29 ± 0.02	47.9 ± 2.3	6.72 ± 0.09	83 ± 0.19	78 ± 0.16	<3
TFT 4	0.28 ± 0.06	48.2 ± 2.2	6.79 ± 0.08	76 ± 0.18	77 ± 0.19	<3

4. Discussion

According to literature, *Legionella* spp. control is needed in hospital settings, mostly in high-risk areas hosting immunosuppressed patients [15,16]. International and Italian guidelines [6,7] recommend

the application of control measures to ensure good microbiological quality in hospital water networks. The choice of an adequate and continuous physical or chemical disinfection system may prevent the occurrence of waterborne pathogen colonization in hospital water plants [17], but the complete eradication of *Legionella* spp. seems mostly improbable if the colonization is high and the water network is old, large, and complex, with critical points [18]. In spite of the age of the water plant and the presence of continuous chlorination in Hospital 3, we detected the presence of high *Legionella pneumophila* concentration in hot water samples. This is due to the low hot water use in the hospital, which caused the persistence of *Legionella* spp. bacteria in biofilm, as described in our preliminary study [12]. In fact, the presence of dead-end branches in water networks allows the biofilm growth in points where the disinfectant cannot be effective against microorganisms [19].

Despite the different number of hospital levels or beds, the amount of dead-end branches detected in hot water networks were the same, with the exception of Hospital 3. In fact, we installed four TFTs in Hospital 1, Hospital 2, and Hospital 4, while seven TFTs were installed in Hospital 3.

The choice of TFT installation points depends on the kind of subjects (patients, healthcare workers, etc.) present in those areas. We avoided TFT installation in stays areas, surgeries, and medical offices in order to limit the exposition to contaminated aerosol during TFT installation.

These devices were mostly placed in storage rooms, kitchens, and locker rooms.

The TFT installation occurred in correspondence with dead-end sections in all the hospitals and the implementation of continuous chemical chlorination in Hospital 1, Hospital 2, and Hospital 4, and the municipal water pre-filtration devices allowed *Legionella pneumophila* decontamination during the period between T1 (24 h) and T4 (one month). As shown in Figure 5, *Legionella pneumophila* colonized all hospitals at T0 and T1. Contaminations persisted in Hospital 1 for one month and in Hospital 3 for seven days, despite the presence of total chlorine concentrations higher than 0.25 mg/L. After two months, *Legionella pneumophila* was no longer detected. Moreover, in all hospitals, the increase of temperature values was obtained from T0 to T1. This is due to the maintenance works on the hot water networks performed during TFT installation. Considering the low hot water temperatures detected at T0 (≤40 °C), we increased this value, in order to achieve boiler water temperatures of at least of 50 °C. According to recent studies, the increase in hot water temperature, lack of water stagnation, and application of good hydraulic practices play a role in *Legionella* spp. control in water networks [20–22].

Our results show that water flushing eases the chlorine dioxide efficacy in water networks. A reduction of stagnant water reduces biofilm growth, which is considered as a form of microorganism resistant against chemical disinfectants, such as chlorine dioxide, applied in water networks [23,24].

Literature data has shown the impact of unsteady flow on drinking water quality. Considering that stagnation promotes bacterial accumulation, special attention should be given to the biological quality of drinking water where consumption is variable. Interactions between hydraulics and biofilm structures are complex, and bacteria may be released from the biofilm into water after only 1 h of stagnation, allowing water quality degradation [22–25].

In conclusion, our study continues the assessment of TFT device applications from our previous work [12]. The aim of the TFTs was to increase the efficacy of the water disinfection system. In fact, TFT installation is considered a good cost-effective method which helps maintain disinfectant levels throughout the system [26]. Although the periods of contamination decrease were long, this method could represent a valid choice for improving drinking water quality and disinfectant efficacy, reducing the critical section of water plants and biofilm maturation in dead-end branches.

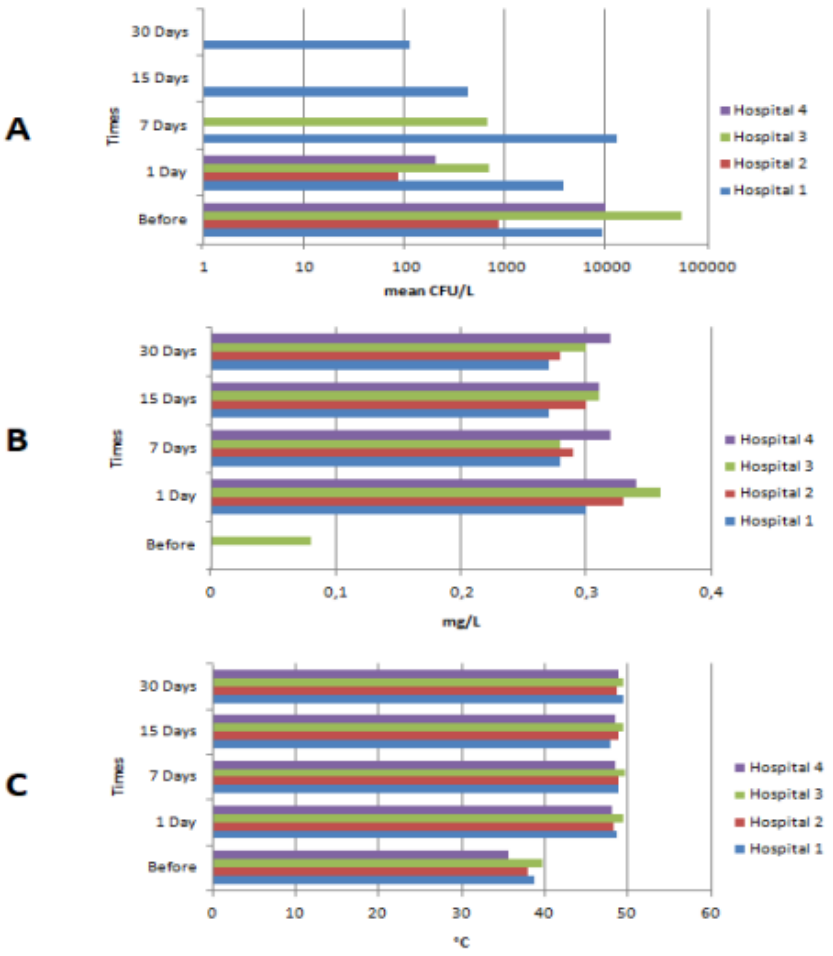

Figure 5. *Legionella pneumophila* counts (CFU/L) (**A**), total chlorine concentration (mg/L) (**B**), and temperature values (°C) (**C**) detected in all the hospitals.

Author Contributions: A.B. and G.P. conceived and designed the experiments. M.T., B.C., P.V. and A.L.C. performed the experiments and wrote the paper. T.M., C.B. and E.D.V. performed the water samplings. All authors have read and agreed to the published version of the manuscript.

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Anexo 6. Capturas del artículo n°6 titulado: Water Safety Plan, Monochloramine Disinfection and Extensive Environmental Sampling Effectively Control Legionella and Other Waterborne Pathogens in Nosocomial Settings: The Ten-Year Experience of an Italian Hospital.

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Article

Water Safety Plan, Monochloramine Disinfection and Extensive Environmental Sampling Effectively Control *Legionella* and Other Waterborne Pathogens in Nosocomial Settings: The Ten-Year Experience of an Italian Hospital

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Abstract: *Legionella* contamination control is crucial in healthcare settings where patients suffer an increased risk of disease and fatal outcome. To ensure an effective management of this health hazard, the accurate application of a hospital-specific Water Safety Plan (WSP), the choice of a suitable water disinfection system and an extensive monitoring program are required. Here, the ten-year experience of an Italian hospital is reported: since its commissioning, Legionellosis risk management has been entrusted to a multi-disciplinary Working Group, applying the principles of the World Health Organization's WSP. The disinfection strategy to prevent *Legionella* and other waterborne pathogens relies on the treatment of domestic hot water with a system ensuring the in situ production and dosage of monochloramine. An average of 250 samples/year were collected and analyzed to allow an accurate assessment of the microbiological status of water network. With the aim of increasing the monitoring sensitivity, in addition to the standard culture method, an optimized MALDI-ToF MS-based strategy was applied, allowing the identification of *Legionella* species and other relevant opportunistic pathogens. Data collected so far confirmed the effectiveness of this multidisciplinary approach: the fraction of positive samples never overcame 1% on a yearly basis and Legionnaires' Disease cases never occurred.

Keywords: *Legionella*; Water Safety Plan; monochloramine; waterborne pathogens; prevention

1. Introduction

Since its debut and subsequent identification during the outbreak of Philadelphia in 1976 [1,2], *Legionella pneumophila* has shown itself as a serious risk for human health [3]. According to current knowledge, the *Legionella* genus includes over 70 species of aerobic, Gram-negative ubiquitous bacteria spread worldwide in both natural water bodies and artificial water distribution systems [4,5]: their growth and proliferation are particularly boosted in the latter engineered habitats, providing favorable water temperatures (25–45 °C), nutrients abundance and surfaces facilitating biofilm formation.

The *pneumophila* species, as well as other species of the genus, are responsible for the pathological conditions collectively known as legionellosis [6–8], comprising both Pontiac

fever, a mild flu-like illness, and Legionnaire's Disease (LD), the pneumonic and more severe form, that frequently lead to fatal outcomes [9,10].

The risk of *Legionella* infection represents a critical issue in particular for hospitals and other healthcare-related buildings [11–13]. Indeed, chronic lung diseases and, more in general, any condition associated with immunodeficiency can significantly contribute to increase individual susceptibility and prognosis severity [9]: patients of healthcare settings are thus generally prone to enhanced vulnerability. In addition, the practice of airways-related clinical procedures can increase the risk of exposure to such a kind of waterborne pathogens transmitted by inhalation of contaminated water droplets [14].

According to European data, the case fatality associated with hospital-acquired and healthcare-related infections, which accounts for the 8% of the total cases, increased from the general rate of 10% to 30–50% [15,16]. Recently, the Italian health authority Istituto Superiore di Sanità (ISS) reported that for 2021, the case–fatality ratio for hospital-acquired cases in Italy was even 40.5% [17].

Given the relevance of this opportunistic pathogen and of its consequences in terms of disease burden, it is no wonder that the Drinking Water Directive 2020 (2020/2184) introduced *Legionella* as a new parameter to be monitored to ensure a proper water quality, especially in the domestic water distribution systems of priority premises, and recommended a risk-based approach for its proper management.

In the last few years, an increasing amount of attention has been also addressed to other waterborne pathogens [18–20], both bacteria and fungi, that, similarly to *Legionella*, may represent a risk for human health, particularly for healthcare patients affected by chronical diseases or by immunocompromised states [21,22].

When dealing with microbiological water safety, it is important to consider that the construction and commissioning stages of a new building are characterized by an increased risk of microbial colonization and biofilm formation in water piping [23–25]: it is thus crucial, especially when dealing with healthcare settings, to rely on an effective strategy for *Legionella* (and other waterborne pathogens) risk management even before the opening of a new building, to properly keep the water distribution system status under control since its installation [26–28].

For this reason, in 2012, when moving from the old 1930s hosting building to a new, 997-bed structure, the Management of Papa Giovanni XXIII Hospital (Bergamo, Italy) appointed a dedicated multidisciplinary Working Group for the definition and implementation of a tailor-made Water Safety Plan (WSP) based on the principles stated by World Health Organization and national guidelines [4,29,30] to ensure a reliable strategy for legionellosis risk management even before commissioning.

The choice of the right water disinfection approach represents a crucial factor for such a strategy: it should ensure satisfactory results in terms of microbiological (and chemical) water safety while being fully compatible with the structure and composition of water distribution systems [31,32]. In this case, monochloramine was chosen for domestic hot water treatment: this chlorine-based disinfectant was used since the beginning of the last century by municipalities for the secondary disinfection of drinking water, but it was at its first applications for *Legionella* prevention in domestic hot water systems at that time [33].

In addition to the establishment of preventive and control measures, the Working Group drew up a strict protocol of self-checking, which was based on an extensive *Legionella* monitoring campaign. As analytical approach, an internal multistep procedure was adopted, combining a culture-based method with the downstream application of MALDI-ToF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) for *Legionella* identification at the species level.

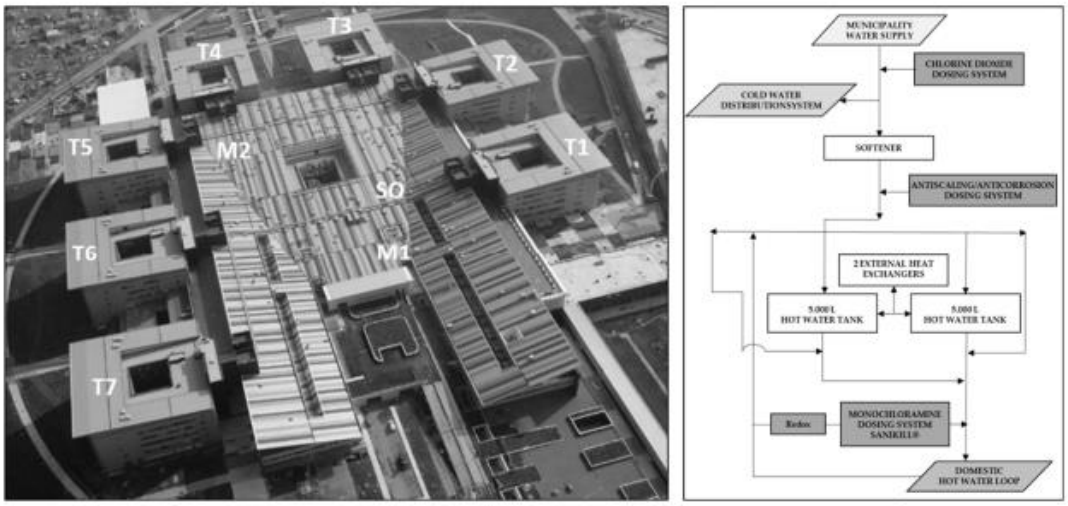
This approach allowed the monitoring not only of *Legionella* species but also of other waterborne opportunistic pathogens of nosocomial relevance, ensuring a more exhaustive control of the microbiological quality of water.

In the present work, the experience of Papa Giovanni XXIII Hospital from commissioning until 2022 is reported, with the aim of proving the importance of prevention and surveillance and the effectiveness of such strategies.

2. Materials and Methods

2.1. Setting

Papa Giovanni XXIII Hospital is one of the biggest healthcare facilities in Italy, with 997 beds distributed over 320,000 square meters. The hospital consists of 7 five-story towers and a three-story main building called "the Plate" (Figure 1). The towers host the wards while the main building hosts the emergency department, surgery rooms, ICUs, outpatient clinics, staff offices and laboratories. The pharmacy and some restaurants, bars and shops are also located at the lobby level.



(**a**) (**b**)

Figure 1. (**a**) Aerial view of the hospital depicting its structure and the positions of the domestic hot water production plants (T1–T7, M1, M2, SO); (**b**) schematic representation of the hot water production plants.

2.2. Piping and Water Treatment

Drinking water supplied by the local municipality (see Supplementary Table S1 for details about chemical features) is distributed to all the hospital buildings after additional filtration and in continuous disinfection with in situ generated chlorine dioxide (Grundfos). Chlorine dioxide concentration in cold water is kept at a residual level of 0.1–0.2 mg/L.

Water piping is mainly made of crimped AISI 316L stainless steel for risers and return loop and PP-r (random polypropylene) plastic for the end distribution from the risers to the outlets of each room.

The Plate drinking water distribution system is made of risers that bring water to each floor and of horizontal loops that distribute water to all the outlets. The Plate is equipped with three hot water production units (named M1, M2, SO). In each unit, hot water is produced on a plate heat exchanger and is accumulated into two in parallel 5000 l storage tanks. A mixing valve assures that hot water is distributed at the right temperature (see below). M1 and M2 work in parallel, and each of them is proportioned to feed all the main building in case of failure of the other one. SO only feeds the staff changing room at level 1 and the morgue. Both M1 and M2 hot water heaters provide water for the main loop at level 1, from which the risers up to level 3 depart. The recirculation loop at level 3

collects all the flows in the riser and directs them toward both the M1 and M2 hot water production units.

Each tower is equipped with an independent domestic hot water production unit (named T1 to T7) that feeds the tower itself and part of the connecting building with the towers that precede and follow. The hot water production unit, placed at level 0, feeds a loop placed at level 1 that brings water to the risers up to level 6. The main recirculation loop collects the water from the risers on level 5. Each hot water loop also supplies half of the connection buildings (5–6 rooms for each floor) between towers at levels 1 and 2. The recirculation loop for these connection buildings is close to level 2 on the main pipe of the recirculation loop that arrives from level 5.

The hot water production stations were originally designed and realized to carry out thermal shock at 75 °C along all the piping system. For this reason, each room (patient rooms, surgery rooms, ICUs, nursing rooms, staff changing rooms, visitor restrooms, etc.) is equipped with a mixing valve to avoid scalding. Within all the ten years of monitoring herein described, water was kept, thanks to an electronic control system, at 50–55 °C in the storage tanks and at 48 °C after the mixing valves.

Hot water is softened to reach 15 °f and then treated with monochloramine produced and dosed by a patented system (SANIKILL, Sanipur SpA, Flero, Italy) specifically developed to treat domestic hot water. The SANIKILL system synthetizes monochloramine directly into the hot water flow, fine tuning the proper N/Cl ratio, and keeping a constant monochloramine concentration thanks to an on-line Oxidation-Reduction Potential (ORP) probe. Dosing systems are settled to keep monochloramine residual concentration at the outlets between 2 and 3 mg/L.

Monochloramine and ammonia concentrations are monthly monitored at designed sentinel points within the buildings by on-site analysis with a modified indophenol method (Monochlor F, HACH) performed with a DR900 colorimeter (HACH).

2.3. Water Safety Group and Water Safety Plan

As discussed more thoroughly in the Section 3, even before its opening, the water safety of the hospital has been assured by an interdisciplinary Water Safety Group (WSG) whose first purpose was to implement an ad hoc Water Safety Plan (WSP). Several meetings were organized before the commissioning to reach this goal. After the opening at the end of 2012 until now, the WSG has met at least 4 times a year.

2.4. Sampling and Microbiological Analysis

Starting from November 2011, one year before its opening, the hospital has been checked for the presence of *Legionella* in drinking cold water and domestic hot water systems by the local health authority.

After the commissioning, an average of 250 samples/year has been regularly collected (one sampling round every 30–60 days) and analyzed by the internal Microbiology and Virology Laboratory (M&V) for the presence of *Legionella* spp. According to WSP requirements, samples were collected from representative points of the domestic hot water distribution systems (e.g., storage tanks, hot water main, recirculating loop, outlets). Starting from 2016, water samples were analyzed also to assess the presence of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Fusarium* spp., and *Aspergillus* spp. to fulfill the improved requirements defined by the WSG for a stricter monitoring of water quality in high-risk clinical units hosting immunocompromised patients.

Samples were collected without flushing and without flaming in accordance with the Italian Guidelines for Legionellosis Prevention. Bacteriological sampling was performed with one-liter sterile bottles supplemented with 1 mL of sodium thiosulphate (10 mg/L) for the neutralization of residual disinfectant.

To ensure the reliability and comparability of the results, a standard procedure for sample collection, transport and storage was established and observed. Samples were delivered to the laboratory for bacteriological analysis immediately after collection.

Samples were processed according to an internal procedure based on ISO 11731-2:2004 (until 2017) and ISO 11731:2017 (after 2017): 500 mL of water were first vigorously shaken and then filtered by pouring the samples into a sterile 0.45 µm nitrocellulose membrane filter with a 50 mm diameter (International PBI, Milano) using a vacuum source in a biological safety cabinet. The membrane filter underwent acid treatment and washing with PAGE solution. It was then aseptically removed from the holder with sterile filter forceps and directly plated onto Buffered Charcoal Yeast Extract (BCYE) Agar (bioMérieux). Plates were incubated at 35 °C + 2 °C in a humidified incubator with an atmosphere of 2.5% CO_2. Cell plates were then examined after 72 h of incubation: the negative ones were re-incubated until 10 days after inoculation. The same procedure was followed with the remaining 500 mL of water sample for the analysis of other waterborne pathogens: in this case, the filter membrane was plated onto Tryptic Soy Agar (bioMérieux), incubated at 35 °C + 2 °C in air and examined after 24 h of incubation. The negative ones were re-incubated for a total of 4 days after inoculation for bacteria and of 7 days for fungi.

Growing colonies were then firstly identified by stereomicroscopy and Gram staining. When necessary, presumptive colonies were previously sub-cultured to obtain pure cultures. Finally, 1–3 colonies were directly examined by the MALDI-ToF MS technique using a VITEK®MS v2.0 (bioMérieux) analytical procedure according to the manufacturer's instruction. Isolated bacterial colonies were applied to a single well of a disposable, barcode-labeled target slide (VITEK® MS-DS) using a 1.0 µL loop overlaid with 1.0 µL of a saturated solution of alpha-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile and 2.5% trifluoroacetic acid (VITEK® MSCHCA) and then air dried. For instrument calibration, an *Escherichia coli* reference strain (ATCC 8739) was transferred to designated wells on the target slide using the procedure described above. The VITEK®MS v2.0 system includes an OEM (original equipment manufacturer)-labeled Shimadzu AXIMA Assurance mass spectrometer linked to a reference database, which is referred to as the Knowledge Base. During target interrogation, mass spectra within a range of 2000 to 20,000 Da were recorded in linear positive mode at a laser frequency of 50 Hz. For each interrogation, laser shots at different positions within the target well producing up to 100 mass profiles were summed into a single, raw mass spectrum. The processed (binned) data were used to query the Knowledge Base to determine the taxonomic identity. Since January 2017 until August 2018, the library version was V.3, including six *Legionella* species and subspecies (*L. anisa*, *L. bozemanae*, *L. feelei*, *L. longbeachae*, *L pneumophila* with ssp. *fraseri* and *pneumophila*). Then, the software version V.3.2 was adopted: this library version includes 18 *Legionella* species and subspecies (*L. anisa*, *L. birminghanensis*, *L. bozemanae*, *L. cincinnatiensis*, *L. erythra*, *L. feelei*, *L. jamestowniensis*, *L. jordanis*, *L. londiniensis*, *L. longbeachae*, *L pneumophila* with ssp. *fraseri* and *pneumophila*, *L. rubrilucens*, *L. sainthelensi*, *L. taurinensis*). When *Legionella pneumophila* was identified, serogroups were determined with an agglutination test (*Legionella pneumophila* antisera set, Biogenetics Diagnostics, Italia).

Simplified criteria were then adopted to determine *Legionella* (and other waterborne pathogens) concentration in CFU/L for this unofficial, internal water microbial control. Colonies were manually counted, and the number of colonies obtained was multiplied by 2 (given that 500 mL of water is used for the analysis) in order to obtain the microbial concentration expressed as CFU/L. When higher bacterial concentrations were detected and if the colonies were homogenously distributed on the plate, given that each filter is divided into 145 squares, the value of CFU/L was calculated as follows:

$$Cs = (a \times 145) \times 2$$

where *Cs* is the value of *Legionella* concentration expressed in CFU/L; *a* is the number of colonies in a square of the filter; 145 is the number of squares in each filter; and 2 refers to the half-liter (500 mL) water-filtered volume.

Starting from 2020, due to the COVID-19 pandemic, the monitoring routine changed by focusing the investigation on *Legionella* and by outsourcing the analysis to a third-party laboratory, which applied the standard ISO 11731:2017 method.

3. Results

3.1. Water Safety Group, Water Safety Plan and Disinfection Strategy

Given the relevance of Legionellosis and other opportunistic pathogen-driven infections in nosocomial environments, since its opening at the end of 2012, after 7 years of construction works, the water safety of the hospital has been assured by an interdisciplinary Water Safety Group (WSG) composed of the Medical Director, Infection Control Team members, Technical Director and technical staff, Hospital Hygiene Unit, M&V Laboratory Director and proven experts in *Legionella* remediation from external firms. The first purpose of the WSG was to perform an accurate risk assessment and consequently implement a Water Safety Plan (WSP) following the indication of the World Health Organization [4] and national guidelines [29,30]. Several meetings were organized before commissioning to reach this goal. Each decision was submitted to the local health authority for comments and approval. After the opening at the end of 2012, until now, the WSG has met at least four times a year to analyze and discuss monitoring results, update the WSP (at least yearly, as well as in case of significant renovation works or of publication of updated national guidelines, as in 2015) and to take any corrective action required to keep water quality at the highest levels.

The WSG performed and periodically revised an in-depth risk assessment, analyzing the characteristics, function and management of each facility and taking into consideration the features of the water distribution systems. Consequently, they defined, and updated when required, a series of control measures to adopt, a set of protocols to follow, a list of parameters to monitor and a microbial sampling program to implement. As an example, starting from 2016, the WSG suggested, in addition to *Legionella* surveillance, the introduction of a stricter microbial water-monitoring routine by requiring the assessment of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Fusarium* spp., *Aspergillus* spp. in high-risk clinical units hosting immunocompromised patients.

Hospital staff were properly trained according to WSP principles and recommendations to actively participate, based on their specific functions, in WSP application by taking care of flushing protocols, maintenance procedures and samples collection.

A crucial step in the definition of reliable control measures to ensure an effective *Legionella* risk management was the choice of a proper strategy for water disinfection. In addition to cold water disinfection with in situ generated chlorine dioxide, the original technical project did not envisage any chemical disinfection for domestic hot water: thermal treatment was proposed for *Legionella* control by keeping the hot water temperature at 65–70 °C in the storage tanks. The risk assessment performed by the WSG highlighted that considering the extension of the hot water distribution systems and given the presence of mixing valves, thermal treatment could be inadequate to effectively avoid *Legionella* proliferation and that the residual chlorine dioxide coming from cold water was obviously insufficient to ensure a proper disinfection.

It was then decided to introduce an additional chemical treatment for domestic hot water: the hypothesis to adopt chlorine dioxide was immediately discarded, given its incompatibility with the PP-r made piping present in the water distribution systems and the unsatisfactory and undesirable outcomes observed in the old building previously hosting the hospital. In that year (2012), as an example, despite the in continuous dosage of chlorine dioxide, 18% of the samples collected (13 out of 72) were positive for *Legionella*.

The WSG decided to adopt an innovative strategy based on monochloramine dosage.

Monochloramine is in situ produced and dosed proportionally to water consumption and residual concentration, keeping 2–3 mg/L at the outlets. Monochloramine concentration is monthly checked at designed sentinel points within the buildings: the data in Table 1 represent for each year the average concentration of the measurements monthly performed at the hot water return loop of each of the ten domestic hot water distribution systems. These data confirmed that the concentration of monochloramine was maintained within the target range (approximately 2.5 mg/L). Concentration values measured at the outlets (were consistent with the data here summarized.

In addition, ammonia concentration was periodically analyzed: as proved by the data reported in Table 1, the disinfection system ensures an adequate minimization of this chemical species. The average ammonia concentration value was always below the limit value of 0.5 mg/L defined by the Italian legislation.

Table 1. Average monochloramine (NH_2Cl) and ammonia (NH_4^+) concentration values (±standard deviation) expressed in mg/L. Data reported are mean values calculated by the measurements monthly performed at the hot water return loop of each of the ten domestic hot water distribution systems.

Year	NH_2Cl (mg/L)	NH_4^+ (mg/L)
2013	2.57 ± 0.35	0.39 ± 0.13
2014	2.50 ± 0.43	0.43 ± 0.08
2015	2.58 ± 0.62	0.34 ± 0.11
2016	2.66 ± 0.21	0.35 ± 0.14
2017	2.56 ± 0.17	0.35 ± 0.11
2018	2.51 ± 0.27	0.37 ± 0.10
2019	2.51 ± 0.25	0.40 ± 0.06
2020	2.53 ± 0.56	0.37 ± 0.06
2021	2.48 ± 0.55	0.33 ± 0.11
2022	2.49 ± 0.46	0.33 ± 0.10

3.2. *Legionella* Control

The microbial pre-commissioning water sampling performed in 2011 by health authorities highlighted a widespread and consistent microbial contamination: 10 of the 20 samples collected were positive for *Legionella* contamination but it was impossible to properly define the concentration because of the presence of abundant interfering microorganisms. For the other six samples, the concentration of microorganisms was so high to actually preclude any further analysis.

At the end of 2012, before the commissioning, remediation protocols based on a hyperdosage of chlorine dioxide (1 mg/L at distal outlets) and monochloramine (5 mg/L at distal outlets) were performed, respectively, on cold and domestic hot water. The shock treatment was effective in restoring safe microbial conditions of the water systems, zeroing the positivity previously identified: after the treatment, *Legionella* was absent in all the samples collected (147 for domestic hot water and 18 for drinking water).

Since then, the hospital has undergone a strict protocol of microbiological self-checking to confirm the efficacy of the measures adopted for legionellosis risk management. This checking protocol has become increasingly accurate and massive in these 10 years of monitoring: in the last years, more than 300 samples were yearly collected from both drinking and domestic hot water, with an average sampling amount referred to the whole monitoring period of more than 250 samples/year. The results of *Legionella* monitoring are summarized in Table 2.

From 2013 to 2015, none of the samples collected was positive for *Legionella*.

Starting from 2016, the culture method has been complemented with the MALDI-ToF MS analysis, allowing the identification of all *Legionella* at species level.

From 2016 to 2019, the yearly percentage of positive samples was always below 2% and rarely the concentration exceeded the 100 CFU/L limit stated by the national guidelines. Thanks to the analytical approach adopted, *Legionella* species other than *pneumophila* were also identified (*L. feeleii*, *L. bozemanii*, *L. anisa*).

It is relevant to note that most of the positive samples were isolated from outlets fed by a minor domestic hot water loop lacking continuous disinfection.

Starting from 2020, due to the emergency status caused by the COVID-19 pandemic, the internal Laboratory of Microbiology and Virology was no longer able to take care of such an extended *Legionella* analysis, which was thus entrusted to an external lab, adopting the standard ISO 11731:2017 method. During 2020 and 2021, the absence of *Legionella*

(<100 CFU/L, according to the analytical method limit) was confirmed for all the samples collected. In 2022, only three positive samples were found: all of them were isolated from the already mentioned minor domestic hot water loop lacking a continuous disinfection system.

Table 2. *Legionella* monitoring data. For each year of the monitoring period, the number of total samples collected is reported and the distribution between domestic hot water (h) and drinking water (c) samples is specified. The number of samples positive for the presence of *Legionella* spp. (i.e., samples with at least 2 CFU/L) are reported (and expressed as percentage with respect to the total of domestic hot water or cold water samples—value within brackets). The number of positive samples showing a concentration value > 100 CFU/L is also specified (and expressed as percentage with respect to the total of domestic hot water or cold water samples—value within brackets).

Year	Total Samples			Positive Samples (%)	Samples > 100 CFU/L (%)
2013	148	h	138	0 (0.0%)	0 (0.0%)
		c	9	0 (0.0%)	0 (0.0%)
2014	183	h	161	0 (0.0%)	0 (0.0%)
		c	21	0 (0.0%)	0 (0.0%)
2015	191	h	171	0 (0.0%)	0 (0.0%)
		c	20	0 (0.0%)	0 (0.0%)
2016	191	h	169	2 (1.2%)	1 (0.5%)
		c	22	0 (0.0%)	0 (0.0%)
2017	259	h	232	4 (1.7%)	1 (0.4%)
		c	27	0 (0.0%)	0 (0.0%)
2018	300	h	264	5 (1.9%)	3 (1.1%) *
		c	36	1 (2.8%)	1 (2.8%)
2019	255	h	210	3 (1.4%) *	0 (0.0%)
		c	45	0 (0.0%)	0 (0.0%)
2020 **	317	h	251	0 (0.0%)	0 (0.0%)
		c	66	0 (0.0%)	0 (0.0%)
2021 **	363	h	296	0 (0.0%)	0 (0.0%)
		c	67	0 (0.0%)	0 (0.0%)
2022 **	344	h	281	3 (1.1%)	3 (1.1%) *
		c	63	0 (0.0%)	0 (0.0%)

(*) Samples collected from outlets fed by a minor domestic hot water loop lacking chemical treatment). (**) Analysis performed by an external laboratory according to standard ISO 11731:2017 method (negative sample: *Legionella* < 100 UFC/L).

3.3. Additional Microbiological Monitoring

The combined culturing/MALDI-ToF MS analytic approach allowed an improvement of microbiological water monitoring by identifying other waterborne pathogens relevant for nosocomial infections. From 2016 to 2019, the periodical monitoring reports have been enriched for all the samples analyzed by data relative to the detection of the Gram-negative bacteria *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* and fungi belonging to *Fusarium* and *Aspergillus* genera. Unfortunately, this additional analysis was significantly reduced during 2020, 2021 and 2022 for the same reasons mentioned above.

Table 3 summarizes the outcome of this monitoring: for each of the pathogens considered, the number of positive samples was negligible with respect to the total number of samples analyzed, and no relevant or alarming variations in these microbial populations were generally observed within the seven years of monitoring. Only a slight increase in the number of *P. aeruginosa* positive samples was recorded in domestic hot water, in particular during 2019 (when also for cold water, the number of positive samples was slightly higher) and 2020, even if the percentage of positivity always remained below 10%. The data collected during the last two years, however, suggest a trend reversal, with a reduction in the number of positive samples.

Table 3. Other waterborne pathogens monitoring. For each year of the monitoring period, the number of total samples collected is reported and the distribution between domestic hot water (h) and drinking water (c) samples is specified (except for 2022 samples). The number of positive samples (i.e., samples in which the presence of at least 2 CFU/L of the pathogen of interest was detected) is expressed as absolute value and as a percentage with respect to the corresponding total of domestic hot water or cold water samples (value within brackets).

Year	Total Samples			*P. aeruginosa*	*A. baumannii*	*S. maltophilia*	*Fusarium* spp.	*Aspergillus* spp.
2016	135	h	124	1 (0.8%)	0 (0.0%)	4 (3.2%)	0 (0.0%)	1 (0.8%)
		c	11	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2017	225	h	196	4 (2.0%)	0 (0.0%)	10 (5.1%)	2 (1.0%)	4 (2.0%)
		c	27	3 (11.1%)	0 (0.0%)	2 (7.4%)	0 (0.0%)	0 (0.0%)
2018	268	h	236	5 (2.1%)	1 (0.4%)	1 (0.4%)	2 (0.8%)	3 (1.3%)
		c	32	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2019	307	h	261	18 (6.9%)	0 (0.0%)	8 (3.1%)	1 (0.4%)	6 (2.3%)
		c	46	5 (10.8%)	0 (0.0%)	4 (8.7%)	2 (4.3%)	4 (8.7%)
2020	180	h	147	12 (8.2%)	0 (0.0%)	2 (1.4%)	0 (0.0%)	0 (0.0%)
		c	33	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2021	14	h	14	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		c	0	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2022	76	/		3 (3.9%)	0 (0.0%)	4 (5.3%)	0 (0.0%)	0 (0.0%)

4. Discussion

As reported by the ECDC, Legionnaires' disease does represent an important cause of potentially preventable morbidity and mortality in Europe [34]. Its prevention should rely on an accurate risk evaluation and on the subsequent application of appropriate control measures, especially in the case of settings that host population subgroups more susceptible and at higher risk. This is the case of hospitals, nursing homes and other healthcare facilities. Indeed, as previously mentioned, the last ISS report clearly showed the harmfulness of this illness and of its outcomes in healthcare related-structures [17].

The new European Drinking Water Directive further confirmed the relevance of *Legionella* and its impact on water quality and human health by introducing it as a new parameter to be monitored in all water distribution systems, with a particular focus on water distribution systems of priority buildings. It also stressed the importance of managing this issue with a risk-based approach that allows effective preventive strategies.

The present work describes the experience in Legionnaires' disease prevention of a new 1000-bed hospital and sharing the encouraging results obtained to prove the effectiveness of the multidisciplinary preventive approach adopted even before its commissioning.

It is indeed well known that construction activities, given their nature and the kind of operations that they may entail, can strongly pose a risk of microbiological contamination the water systems of new buildings (as maintenance works can in pre-existing ones), contributing to the growth and spread of waterborne pathogens in the system itself [35,36]. Not surprisingly, cases of nosocomial Legionellosis attributable to construction or maintenance works have been previously reported [23].

For this reason, the Water Safety Group that takes care of *Legionella* risk management started to meet before commissioning to draft a comprehensive Water Safety Plan: they performed a deep evaluation of all the critical issues to be considered when taking care of *Legionella* control in such a complex building and they subsequently defined critical control points and a complete repertoire of preventive and corrective actions to avoid the occurrence of legionellosis cases, according to what reported in previous studies [15,37]. The supervising function of this group, collecting different expertise, plays a crucial role to ensure a constant improvement of risk assessment and a consequent suitable update of the WSP. Similarly, the coordinated activities of a properly trained and informed technical staff (both internal and external) turned out to be essential to effectively put in place preventive and corrective actions. By applying WHO guidelines [4], the management and operating

routine adopted since 2012 largely anticipated most of the principles of the risk-based approach invoked by Directive (EU) 2020/2184 and required according to the latest Italian legislation concerning drinking water (D. Lgs. 18/2023).

The choice of the suitable disinfection approach was another key point of the strategy adopted for Legionellosis prevention. The thermal treatment originally suggested in the technical project of the hospital was excluded by the WSG because it was considered ineffective: several studies indeed recently confirmed that thermal treatment cannot be considered a reliable approach to prevent *Legionella* proliferation [38], since it frequently results in regrowth phenomena commonly accompanied by the emergence of viable but nonculturable and thermo-tolerant strains [39,40].

Based on these considerations, the adoption of a supplemental and continuous disinfection strategy for domestic hot water was preferred. The severe problems faced in the former city hospital in terms of both disinfection inefficacy and corrosion issues led to the exclusion of chlorine dioxide. Given its high reactivity and its gaseous nature, it has a reduced half-life, especially at high temperature, where its concentration suffers a rapid decrease [41]. It is thus challenging to keep a proper residual disinfection, especially in complex and long water distribution systems. Moreover, its strong oxidizing properties makes it extremely corrosive for both metallic and plastic pipes [33,42,43] with dramatic consequences in terms of leakages and failures [44]. It was even shown that corrosion reactions account for most of chlorine dioxide consumption in hot water systems [45].

The WSG thus chose to rely on a disinfection strategy based on the dosage of monochloramine: at that time, despite being internationally approved [4,46] and largely applied for over a century by the US and Canada municipalities as secondary disinfectant for drinking water, it was just at the dawn of its use for *Legionella* control. Nevertheless, it was noteworthy and documented that buildings supplied by municipalities with monochloramine-treated drinking water experienced a reduction in *Legionella* contamination and in hospital-acquired Legionnaires' disease incidence in comparison with buildings fed by chlorinated water [47–49]. Moreover, a study reporting the remarkable results obtained with the first experience of monochloramine application for *Legionella* remediation in the domestic hot water distribution system of a hospital was published just the year before, in 2011. [33].

According to its chemical structure, monochloramine is a weak oxidant [50] that is able to specifically react with some amino acids within microbial proteins [51], thus lethally impairing essential metabolic processes and leading to cell death. Its low reactivity and subsequent enhanced stability enable it to persist within water distribution systems, ensuring a suitable residual disinfection at distal outlets, and to penetrate biofilm [52], quantitatively reaching its deep, actively proliferating layers: here, it effectively exploits its biocidal activity against *Legionella* and other waterborne microorganisms. Given its moderate redox potential, it also ensures compatibility with both plastic and metallic pipes. Indeed, in the case here described, neither pipe failures nor corrosion issues have been reported so far despite the ten-year long uninterrupted treatment.

In terms of disinfection efficacy, our results are in line with what was previously reported by several authors in terms of monochloramine efficacy on *Legionella* control [33,37,53–57]. Although this study cannot prove its remediation properties thanks to the initial shock treatment, based on our observations we can state monochloramine effectiveness in ensuring a long-term control of *Legionella* proliferation, as demonstrated for the first time by a decade-long application experience. This was confirmed by the observation that most of the positive samples identified over ten years were collected from outlets fed by a minor domestic hot water loop lacking monochloramine disinfection. According to its specific risk assessment, the introduction of a dedicated disinfection system has been considered as not a priority so far, and the rare positive cases were handled with minor corrective measures. Nevertheless, in accordance with the aim of control improvement of the WSP, the installation of a continuous disinfection system also on this domestic hot water loop is currently under evaluation.

The additional microbiological data collected also prove monochloramine effectiveness in keeping other waterborne pathogens of nosocomial relevance under control and

ensuring a good microbial quality of water, as already demonstrated by other similar experiences [56,57]. In addition to the waterborne pathogens considered in the present studies, increasing concern is emerging about non-tuberculous mycobacteria (NTMs) and the infections that they can cause [58]. The WSG already took these microorganisms into consideration and included them in the analytical repertoire of microbial monitoring some years ago, but the COVID-19 emergency forced them to abandon the analytical approach based on MALDI-ToF MS and to focus mainly on *Legionella* monitoring. The WSG is now planning to restore the previous analytical approach and to include NTMs in the list of microorganisms to be investigated.

This parameter can be particularly relevant if considering that some evidence highlighted a reduced susceptibility of these microorganisms to monochloramine [59]. The literature on this topic is actually controversial: indeed, other authors [57], while studying the same monochloramine generator system here adopted, reported by contrast a reduction in NTMs after monochloramine treatment. Similarly, other data [60] seem to suggest that by keeping a proper monochloramine concentration (higher than 2 mg/L, as was the case here described), it is possible to avoid any unintended NTMs proliferation and even eradicate previous contamination.

As for water safety from a chemical point of view, previous studies demonstrated that the choice of monochloramine ensures the minimization of disinfection by-products typically associated with chlorine-based treatment, such as THM [57,61]. Given that monochloramine is produced by the reaction between chlorine and ammonia precursors, attention should be paid to possible nitrogen-derived by-products like ammonia, nitrites and nitrates, as well as to N-nitrosamines: these by-products are mainly formed when the stoichiometry of the chemical reaction is not properly controlled. It was indeed well documented that when adopting a suitable technology for monochloramine in situ production, all these by-products are produced at negligible levels [37,56,57,61]. The ammonia concentration monitoring here performed confirmed that its concentration was always well below the limit of 0.5 mg/L stated for drinking water by the Italian legislation.

In addition to the choice of a proper disinfection strategy, one of the major strengths of the preventive approach here adopted is the extensive microbial monitoring: the routinely collection and analysis of such a great number of samples enables an extensive and accurate control of the microbiological quality of water and improves our intervention capacity.

The analytical method here adopted also allows an enhanced sensitivity and thus an improvement with respect to the limits of the traditional standard cultural method. In this way, despite the Italian Guidelines for Prevention and Control of Legionellosis defining a threshold value of 100 CFU/L, more stringent control criteria were internally adopted: even a few units of *Legionella* per liter are taken into consideration and reported, allowing proper and prompt corrective actions. Our method for *Legionella* detection and enumeration ensures a theoretical detection limit of 2 CFU/L, enabling us to carry out remediation measures (like enhancing flushing procedure at critical outlets) well before the contamination can reach alert levels that officially require corrective actions according to the national guidelines.

The choice to adopt an analytical strategy that combines a culture method and MALDI-ToF MS characterization contributed to minimizing *Legionella* related risks. In the last two decades, this protein-based recognition technique has been successfully exploited to easily and quickly identify bacteria and other microorganisms [62–64]: it represents a valuable alternative to the conventional phenotypic, biochemical and molecular methods, which is also thanks to its reliability and the remarkable cost-effectiveness.

Previous studies already highlighted all the advantages of choosing this kind of analytical approach proving its effectiveness in the identification of *Legionella* at the species level [65,66]: given the possibility of recognizing a specific peak profile for each species, this approach not only represents a valuable alternative to the traditional identification methods but also broadens the analytical performance, pushing it beyond the simple distinction between *Legionella pneumophila* and *Legionella* spp. and ensuring the possibility of discrimi-

nating within the different species of this genus. The MALDI-ToF MS analytical potential was also demonstrated by a recent study describing the promising results obtained in the comparison of the MALDI Biotyper system with culture and gene sequencing techniques for its application in clinical and environmental *Legionella* surveillance [67]. It is indeed crucial to consider that *Legionella* species other than *pneumophila* can be responsible for human infections [68,69]; the ability to identify them is consequently a powerful instrument to recognize and control possible clusters otherwise neglected.

The present study represents one of the first examples of the routine use of this technique to monitor *Legionella* contamination in hospital settings [70]: our experience confirmed it as a reliable and cost-effective tool that is faster and easier with respect to others that allow for species identification.

As demonstrated by the data collected, this analytical approach not only enables an improvement of *Legionella* monitoring but also broadens the microbiological surveillance capability by including the other waterborne opportunistic pathogens previously discussed, increasingly taken into consideration for their health-risk potential [71].

5. Conclusions

The WHO reported that when considering the health consequences of waterborne pathogens in the European Union, *Legionella* is responsible for the highest health burden. This is the reason why the New Drinking Water Directive, which came into force on January 2021, introduces the monitoring of this microbiological parameter within the assessment of the potential risks stemming from domestic distribution systems. With the general aim of making the water increasingly safer, it reinforces the principles already stated in the specific guidelines for *Legionella* prevention, stressing the importance of a proper management of the corresponding risk especially for those considered "priority premises", which is a category that obviously includes hospitals and healthcare-related facilities.

The present study, by reporting the long-term effects of *Legionella* prevention measures adopted by a large and complex hospital since its commissioning, proves indeed the importance of relying on an effective and comprehensive Water Safety Plan. It also confirms the adoption of the continuous dosage of monochloramine as an effective strategy to prevent the proliferation of *Legionella* as well as of other waterborne pathogens of nosocomial concern. The choice of an extensive sampling approach that takes advantage of the analytical potential of the MALDI-ToF MS strategy provides for an accurate and timely control of the microbiological status of water, improving the capacity of safeguarding the health of patients and staff and preserving the hygienic conditions of water distribution systems. This method confirms itself as a powerful tool for the routine microbiological monitoring of water that could thus be further extended to comprise the surveillance of other potentially harmful microorganisms, such as mycobacteria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11071794/s1, Table S1: Physical–chemical properties of municipal drinking water supplied to the Hospital; Table S2: Domestic hot water consumption.

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Anexo 7. Capturas del artículo n°7 titulado: Environmental surveillance of *Legionella pneumophila* in distal water supplies of a hospital for early identification & prevention of hospital-acquired legionellosis.

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Environmental surveillance of *Legionella pneumophila* in distal water supplies of a hospital for early identification & prevention of hospital-acquired legionellosis

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***Background & objectives*: *Legionella pneumophila*, a ubiquitous aquatic organism is found to be associated with the development of the community as well as hospital-acquired pneumonia. Diagnosing *Legionella* infection is difficult unless supplemented with, diagnostic laboratory testing and established evidence for its presence in the hospital environment. Hence, the present study was undertaken to screen the hospital water supplies for the presence of *L. pneumophila* to show its presence in the hospital environment further facilitating early diagnosis and prevention of hospital-acquired legionellosis.**

***Methods*: Water samples and swabs from the inner side of the same water taps were collected from 30 distal water outlets present in patient care areas of a tertiary care hospital. The filtrate obtained from water samples as well as swabs were inoculated directly and after acid buffer treatment on plain and selective (with polymyxin B, cycloheximide and vancomycin) buffered charcoal yeast extract medium. The colonies grown were identified using standard methods and confirmed for *L. pneumophila* by latex agglutination test.**

***Results*: About 6.66 per cent (2/30) distal water outlets sampled were found to be contaminated with *L. pneumophila* serotype 2-15. Isolation was better with swabs compared to water samples.**

***Interpretation & conclusions*: The study showed the presence of *L. pneumophila* colonization of hospital water outlets at low levels. Periodic water sampling and active clinical surveillance in positive areas may be done to substantiate the evidence, to confirm or reject its role as a potential nosocomial pathogen in hospital environment.**

Key words Environmental surveillance - hospital-acquired pneumonia - hospital water outlets - *Legionella pneumophila*

Legionella pneumophila is a ubiquitous bacterium commonly found in various natural and human-made aquatic environments. They can enter and multiply in hospital water systems in low or undetectable numbers and may result in the acquisition of infection by patients through aspiration of contaminated water or direct inhalation of aerosols. Water outlets, faucets, showers, humidifiers, respiratory devices and nebulizers that have been filled or cleaned with tap water have been reported as potential sources of infection in several cases[1].

Establishing diagnosis of *Legionella* infection considering clinical criteria alone is difficult and warrants some index of suspicion and laboratory diagnosis[2]. This index of suspicion can be raised by generating knowledge about the possible presence of the organism in the hospital water supplies and outlets[3]. *Legionella* colonization of hospital water supplies in >30 per cent of the sampled water outlets merits initiation of specialized laboratory tests for *Legionella* screening in all patients with hospital-acquired pneumonia[3]. There are only a few studies on environmental colonization by *Legionella* from India[4-7]. Considering this, the present study was planned to identify potential environmental sources of *Legionella* species in a tertiary care hospital so that necessary interventions related to early diagnosis and prevention of hospital-acquired legionellosis can be initiated.

Material & Methods

The study was conducted in the department of Microbiology of All India Institute of Medical Sciences, Raipur, a tertiary care hospital in central India over a period of two months from July to August 2016. The study protocol was approved by the Institutional Ethics Committee.

Thirty water outlets in various patient care areas of the hospital were sampled. From each outlet, water samples and swabs from the inner portions of the outlet pipe were collected amounting for a total of 60 samples. Swabs were collected before water samples from the same source.

The samples were processed as per the protocol[8] given by Centers for Disease Control (CDC), Atlanta. To describe briefly, filtrate of water samples and swabs were inoculated after acid buffer treatment on buffered charcoal yeast extract (BCYE) agar with and without antibiotics (polymixin B, cycloheximide, vancomycin). Cultures were examined at intervals until 14 days of incubation. Colonies of *Legionella* were identified as per the standard protocols[8]. Faintly stained Gram-negative pleomorphic bacilli (Figure) which failed to grow on blood agar or L- cysteine-free agar on subculture were suspected for *Legionella* species. The identification was further confirmed by latex agglutination against *L. pneumophila* antisera 1 and 2-15 by commercially available kit (LK04-HiLegionella Latex Kit; Himedia, Mumbai, India).

The isolates which were suspected to be *Legionella* but failed to agglutinate by any of the antiserum were subjected to identification by matrix-associated laser desorption ionization-time of flight (MALDI-TOF) at Post-graduate Institute of Medical Education and Research (PGIMER), Chandigarh.

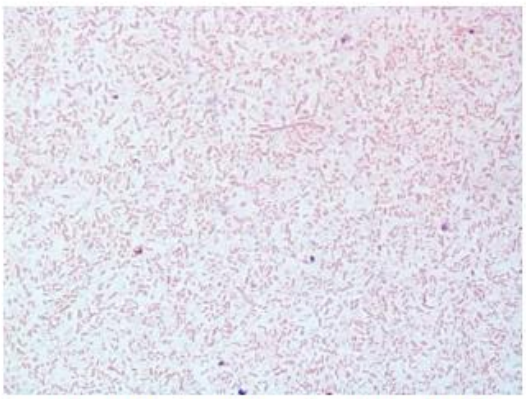

Figure. Gram-stained morphology of the isolate identified as *Legionella pneumophila* the figure shows pleomorphic Gram-negative rods after Gram staining using safranine as a decolourizer for prolonged time (10 min) (×1000).

Statistical analysis: Results were calculated in terms of percentage positivity of the water outlets tested positive for *Legionella*. The proportions were compared using Fisher's exact test.

Results & Discussion

Samples including one water and four swabs, each collected from five (16.6%) different sites (Table), grew colonies suspected of *Legionella* species. However, only two (both from swabs) of these could be confirmed as *L. pneumophila* serotype 2-15 giving percentage positivity of 6.66 per cent (2/30). None of the isolates was identified as *L. pneumophila* serotype 1. The remaining isolates tested by MALDI-TOF were identified as *Reyranella massiliensis*. The swabs yielded more number of organisms as compared to water samples.

Selective BCYE alone could inhibit the growth of environmental bacteria other than *Legionella* in 53.33 per cent (16/30) of water samples and 30 per cent (9/30) of swabs. Acid buffer treatment was able to limit the number of organisms up to 30 per cent (9/30) on plain BCYE agar (P<0.001). It was useful to limit the growth of organisms in 70 per cent (21/30) samples on BCYE with added antibiotics (P<0.001). Acid buffer treatment resulted in further growth inhibition of the *L. pneumophila* isolates.

Table. Hospital water outlets which were suspected for the presence of *Legionella* species

Sl. No.	Name of the site	Type of water source	Nature of sample	Organism isolated and identified as
1	Pre-operative room	Sink tap (number II)	Swab	*Legionella pneumophila* serotype 2-15
2	NICU	Sink tap	Swab	*Legionella pneumophila* serotype 2-15
3	Male medical ward	Sink tap	Swab	Non-*Legionella* species
4	Male medical ward	Water cooler	Swab	Non-*Legionella* species
5	Trauma and emergency ward	Water cooler (number II)	Water sample	Non-*Legionella* species

NICU, neonatal intensive care unit

Routine environmental surveillance for *L. pneumophila* has been a matter of debate over the years. The CDC does not support routine environmental culture for *Legionella* species in the absence of recognized disease, with the exception of transplant unit, because of the supposedly ill-defined relationship between the presence of the organism in the water system and risk of acquiring the infection[9]. However, this approach was countered by different prospective studies across the world in which cases of hospital-acquired legionellosis were discovered subsequent to the identification of *Legionella* colonization of the hospital water supply[10-17].

The present study reported positivity of 6.66 per cent for *L. pneumophila* which was less as compared to the previously reported positivity of 76 and 33 per cent from Indian hospitals[4,7] as well as in the western literature 38 per cent (range 5-83%)[2], 37[18] and 63 per cent[19]. Some of the studies have reported positivity rate of <30 per cent (12-18.7%)[20,21] or even zero[6]. The positivity cut-off of >30 per cent has been used and confirmed by many researchers to indicate the extent of colonization by *L. pneumophila* in any hospital warranting active clinical surveillance of legionnaire's disease[3,11,21]. Colonization in only 6.66 per cent of water outlets obtained in our study does not warrant active screening for the legionnaires' disease in the hospitalized patients. On the contrary of highest reported serotype 1 of *L. pneumophila*, we isolated serogroup 2-15 in our study that has been equally reported worldwide[2,18,19].

Sampling directly the biofilms using swabs was considered better compared to water in increasing the yield of isolation[3,4]. Acid buffer treatment of samples was effective in reducing the contamination; although, it was found to be detrimental to the growth of *L. pneumophila*. Hence, sampling of biofilms without acid buffer treatment, streaked on selective BCYE agar may be considered as the most sensitive method for recovering *Legionella*.

Quantification of environmental samples was tried on water samples by measuring the number of colony forming unit/ml of water cultured as doing so may provide information crucial for assessing the risk of transmission and identifying impending outbreaks. However, it did not prove much useful in our study because on most of the occasions, water culture grew confluent growth of mixed bacteria, making it very difficult to quantitate the slowly growing *Legionellae*. Another limitation of our study was that we did not include hospital-acquired pneumonia cases. Therefore, it was not possible to establish an association between the isolation of *Legionellae* with hospital-acquired pneumonia.

The extent of *Legionella* colonization in hospital water outlets was found to be very less precluding the necessity of incorporating specialized *Legionella* diagnostics in the routine workup of patients with hospital-acquired respiratory infections. However, repeated periodic environmental sampling and active case finding for hospital-acquired legionellosis in areas with positive reports need to be undertaken to confirm or reject its role as a potential nosocomial pathogen in hospital environments.

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***Conflicts of Interest*:** None.

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