# Quantification and identification of aerobic bacteria in holy water samples from a German environment

Christoph König, Stephanie Tauchnitz, Heike Kunzelmann, Christian Horn, Frithjof Blessing, Matthias Kohl and Markus Egert

## ABSTRACT

Despite its frequent use in many religious institutions, the microbiological quality of holy water is clearly underinvestigated. We analyzed the microbial load of 54 holy water samples, repeatedly taken in five Roman Catholic churches in the greater area of Villingen-Schwenningen, Germany, by means of aerobic colony counting and Matrix-Assisted Laser Desorption/Ionization (MALDI) Biotyping of representative isolates. Over all samples, colony counting indicated an average aerobic microbial load of $5.85 \pm 3.98 \times 10^3$ colony forming units (CFU) $ml^{-1}$ (average ± standard error of the mean (SEM)). Urban churches showed significantly higher contaminations than rural churches, probably owing to a greater number of visitors. Out of 145 bacterial isolates, 63 (43%) were identified to genus level and 39 (27%) to species level. The majority of the identified bacteria were typical human skin commensals, mainly affiliated with the genus *Staphylococcus*. Ten out of 20 (50%) of the identified species were classified as potential pathogens. Appropriate hygiene measures should be taken to control microbial contamination of holy water, e.g., regular water exchange, particularly in highly frequented churches.

**Key words** | aerobic bacteria, bacterial load, holy water, hygiene, MALDI Biotyping

**Christoph König**
**Stephanie Tauchnitz**
**Heike Kunzelmann**
**Frithjof Blessing**
**Matthias Kohl**
**Markus Egert** (corresponding author)
Faculty of Medical and Life Sciences, Institute of Precision Medicine,
Furtwangen University,
Jakob-Kienzle-Str. 17,
78054 Villingen-Schwenningen,
Germany
E-mail: *markus.egert@hs-furtwangen.de*

**Christian Horn**
**Frithjof Blessing**
Institute for Laboratory Medicine,
Virchowstr. 10c,
78224 Singen,
Germany

## INTRODUCTION

Holy water is regarded as a symbol of blessing and purification and plays an important role in many ceremonies and customs in Christianity and other religions. For instance, Roman Catholic believers wet their fingers with holy water when entering a church and make the sign of the cross; occasionally they also cross themselves on the forehead, lips and chest. In addition, holy water is used to bless items, food or persons by sprinkling them. Maybe also owing to its religious importance, only a few studies have addressed the microbiological and hygienic properties of holy water so far.

Holy water was first recognized as a source for potentially pathogenic microorganisms in 1992, when *Pseudomonas aeruginosa* was isolated from infected wounds of a hospital patient after sprinkling with holy water (Greaves & Porter 1992). In two other cases, nosocomial infections with *Acinetobacter baumanii* were traced back to contact with holy water (Rees & Allen 1996; Michel *et al.* 2013). Daschner (1997) reported massive contamination with *Pseudomonas* spp. in holy water samples from churches and hospitals in Freiburg, Germany. In several Buddhist temples in Thailand, holy water was examined for microbiological contamination, and the presence of *Escherichia coli* was demonstrated (Phatthararangrong *et al.* 1998). High contaminations with coliform bacteria and members of the *Enterobacteriaceae* family, including a considerable diversity of potentially pathogenic bacteria, were also reported from Spain (Jurado *et al.* 2002). Recently, a landmark study by Kirschner *et al.* (2012) reported contaminations of holy water fonts and holy springs in Vienna, Austria, with cell counts of up to $10^7$ colony forming units (CFU) $ml^{-1}$, and the presence of fecal indicator bacteria such as enterococci and *E. coli*.

Aiming at a deeper understanding of the hygienic relevance of holy water, the goal of our study was to

doi: 10.2166/wh.2017.026

characterize the quantity and diversity of cultivable, aerobic bacteria in holy water samples from Roman Catholic churches in our local environment, and to identify potential factors that drive microbial contamination there. We believe that the data will help to improve the hygiene management of holy water.

## METHODS

### Holy water sampling and colony counting

Fifty-four holy water samples were taken from five different Roman Catholic churches in the greater area of Villingen-Schwenningen, Germany, between May and July 2015. Over this period, samples were collected three times independently from the holy water fonts of the five churches, i.e., from 18 holy water fonts in total (four fonts each were sampled in churches C1-3 and C5, and two fonts in church C4). From each font, 10 ml of water were taken with a sterile syringe (Henry Schein, Melville, USA), after thorough mixing. Water temperature and electrical conductivity (WTW LF 92, Weilheim, Germany) and pH (Five Easy, Mettler Toledo, Columbus, USA) were measured. In addition, building material and hygienic status (corrosion, visible contaminations, etc.) of the sampled fonts were documented. Water samples were immediately put on ice and further processed within 1 h after sampling. After decimal dilution up to $10^{-2}$ with sterile tap water, 100 µl of each dilution were spread in duplicate on tryptic soy agar plates (TSA, Carl Roth Karlsruhe, Germany) and incubated under aerobic conditions for 72 hours at 37 °C. Subsequently, all grown colonies were counted. Only plates with 3–300 colonies were counted. From the counted plates per sample, one representative of each colony morphotype (differing in colony size, color and/or morphology) was picked, T-streaked on a TSA plate, and incubated aerobically at 37 °C overnight. After control of purity, one colony was picked from each morphotype plate, suspended in nuclease free water, and stored at −80 °C for subsequent identification by Matrix-Assisted Laser Desorption/Ionization (MALDI) Biotyping.

In addition to the triplicate sampling of each holy water font per church, the fonts of two churches were sampled directly before and after a holy mass to investigate the direct influence of the churchgoers on the total colony count.

### Identification of representative isolates by MALDI Biotyping

Identification of the obtained bacterial isolates was performed by means of MALDI-Time of Flight (MALDI-TOF) analysis using a MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. The ethanol/formic acid sample preparation procedure was used to increase the efficiency of protein extraction from the bacterial colonies. 1 µl of each protein extract sample was applied onto the spots of the Biotyper steel target. After air drying, the samples were overlaid with 1 µl of alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics), again air dried, and then measured. The obtained fingerprint profiles were matched against the internal MALDI Biotyper reference library (software version 3.3.1.0, containing 5672 bacterial species). Similarities were expressed as score values ranging from 0.0 to 3.0. Scores $\geq$1.7 indicated secure genus identification, scores $\geq$1.9 secure genus and probable species identification (Khot *et al.* 2012).

### Statistical analyses

Statistical analyses were performed using R 3.3.2 (R Core Team 2016). Non-parametric tests (Kruskal-Wallis rank sum test followed by Wilcoxon-Mann-Whitney post hoc tests) were used to check for statistical significance between the colony counts of the five churches. $P$ values $<0.05$ were considered to indicate statistical significance. Additionally, Microsoft Excel (Microsoft Corporation, Redmond, USA, 2016) was used to screen for a correlation between log-transformed colony counts and the numbers of congregants per church.

## RESULTS AND DISCUSSION

### Bacterial load of holy water samples

All investigated samples (100%) showed microbial growth. The bacterial colony counts spanned five orders of

magnitude ($10^0$–$10^4$ CFU ml$^{-1}$; Figure 1(a)) and the average colony count over all 54 samples was $5.85 \pm 3.98 \times 10^3$ CFU ml$^{-1}$ (average ± standard error of the mean (SEM)). A previous study from Vienna, partly using similar cultivation conditions (rich medium, incubation at ~37 °C), reported much higher levels of contamination of holy water fonts in churches with up to $10^7$ CFU ml$^{-1}$ (Kirschner *et al.* 2012). Clearly, the fonts investigated here were less contaminated.

Statistical analyses (Kruskal-Wallis rank sum test) revealed significant differences ($p = 0.023$) among the five churches, and these significant differences occurred between urban churches with more than 3,000 congregants

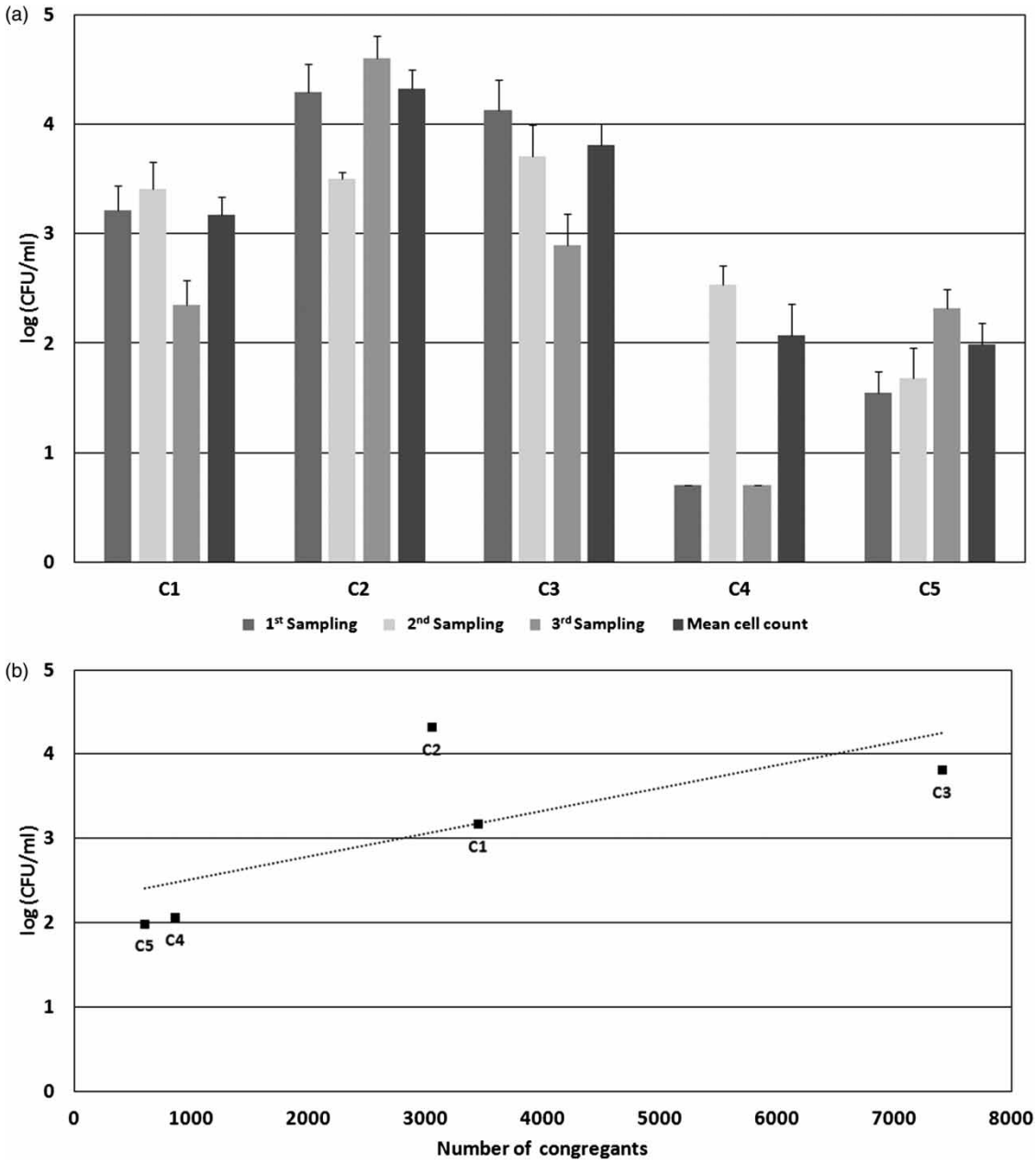


**Figure 1** | (a) Aerobic colony counts in holy water samples of five churches (C1–C5). Values represent mean counts of three independent samplings per church (based on two to four holy water fonts per church) and the overall average per church. Error bars indicate the SEM. (b) Correlation between aerobic colony counts and the numbers of congregants of the investigated churches ($R^2 = 0.5115$).

(C1–C3), and rural churches with less than 900 congregants (C4–5; Wilcoxon-Mann-Whitney test; $p = 0.0026$). A subsequent correlation analysis (Figure 1(b)) suggested a weak linear correlation between log-transformed colony counts and the number of congregants per church ($R^2 = 0.51$), however, the sample size of our study was too small to prove statistical significance for this correlation. Admittedly, the number of actual churchgoers might have been a better parameter for correlation analysis than the number of congregants that belong to the communities of the investigated churches; however, these numbers were not available. In addition, identifying and focusing on the most frequently used water fonts per church, instead of including all sampled fonts per church in the analysis, might have yielded better correlation data. Subsequent analyses should account for that. Finally, a regular exchange of holy water might also explain differences in bacterial load between churches and sampling dates. However, at least for C1 and C3–5 a regular water exchange on a similar (weekly) basis is officially recommended, but not controlled (oral communication of the respective community); a similar recommendation for C2 is likely.

The measured physical and chemical parameters were very stable over the period of investigation and showed no correlation with the colony counts. Water temperature ranged from 18.7 °C to 22.2 °C (average ± SEM: 20.3 ± 0.4), pH-values from 8.2 to 8.4 (8.3 ± 0.0), and electrical conductivity from 0.5 to 1.35 mS cm$^{-1}$ (1.0 ± 0.1), thereby strongly resembling data previously reported from Vienna (Kirschner *et al.* 2012). Conductivity values were higher than average values reported by the local drinking water supplier for the region, i.e., ~0.18–0.45 mS cm$^{-1}$ (online accessible in German via https://www.svs-energie.de), and probably result from salt (sodium chloride) addition, which is a non-standardized key step in the production process of Roman Catholic holy water (Kirschner *et al.* 2012). Neither microbial load nor the physicochemical properties of the water samples showed any correlation to the building material of the holy water fonts or its hygienic status. Finally, colony counts of samples taken before and directly after a holy mass showed no significant differences ($p = 0.753$). The bacterial load in C1 changed from $1.13 \pm 0.74 \times 10^2$ CFU ml$^{-1}$ before to $1.02 \pm 0.57 \times 10^2$ CFU ml$^{-1}$ after mass, and in C3 from $7.95 \pm 7.30 \times 10^2$ CFU ml$^{-1}$ to $8.99 \pm 7.40 \times 10^2$ CFU ml$^{-1}$ (average ± SEM, $n = 4$, respectively).

## Identification of bacterial isolates by MALDI Biotyping

In this study, 145 bacterial isolates were obtained from 54 holy water samples and analyzed with the MALDI Biotyper system (Table 1). For 63 isolates (43%), identification scores ≥1.7 were obtained, allowing secure genus identification. For 39 isolates (27%) the identification scores were ≥1.9, and allowed probable species identification (Khot *et al.* 2012). Based on the identification results, the aerobic cultivable holy water microbiota was dominated by the three bacterial phyla *Firmicutes, Actinobacteria* and *Proteobacteria,* which also represented 94% of all phylotypes identified in a large-scale molecular study on the human hand microbiota (Fierer *et al.* 2008). The holy water microbiota was dominated by Gram-positive bacteria usually associated with humans, in particular staphylococci (30 out of 63 identified isolates, 47%). Characteristic environmental, water-borne bacteria, such as *Sphingobium* spp., were found less frequently.

Staphylococci are typical skin bacteria (Egert & Simmering 2015) and probably originate from the hands of the holy water users. They are well-known for their salt tolerance, which is used for a selective cultivation on Chapman agar (Götz *et al.* 2006). Interestingly, staphylococci are also known to occur in watery habitats (Götz *et al.* 2006), and in the case of the holy water samples they might gain selective growth advantage from the added salt. However, further studies are needed to evaluate survival and (potential) growth of staphylococci and other skin bacteria under holy water conditions.

In line with previous studies (Rees & Allen 1996; Jurado *et al.* 2002; Kirschner *et al.* 2012), we also detected bacteria of probable fecal origin, i.e., enterococci and enterobacteria, in the holy water samples, albeit at a low frequency. For instance, only one out of 54 investigated samples was contaminated with *Enterococcus faecalis,* which is commonly known as a commensal of the human intestine. Based on the German Rule for Biological Agents #446 (German Federal Institute for Occupational Safety and Health (BAuA) 2015), 10 out of 20 species identified here are categorized as risk group 2 (RG 2), i.e., classified as potentially

**Table 1** | Bacterial isolates obtained from holy water samples and identified by MALDI Biotyping with identification scores ≥1.7 (genus level; $n = 63$) and ≥1.9 (species level; $n = 39$)

| Phylum | Family | Genus | Species | Frequency |
|---|---|---|---|---|
| *Firmicutes* | *Staphylococcaceae* | *Staphylococcus* | sp. | 8 |
| | | | *epidermidis*[a] | 8 |
| | | | *warneri* | 2 |
| | | | *pettenkoferi*[a] | 2 |
| | | | *aureus*[a] | 1 |
| | | | *hominis*[a] | 4 |
| | | | *cohnii* | 1 |
| | | | *auricularis* | 1 |
| | | | *pasteuri*[a] | 1 |
| | | | *capitis* | 2 |
| | *Bacillaceae* | *Bacillus* | *cereus*[a] | 1 |
| | *Enterococcaceae* | *Enterococcus* | *faecalis*[a] | 1 |
| *Actinobacteria* | *Micrococcaceae* | *Micrococcus* | *luteus* | 4 |
| | | *Kocuria* | *kistinae* | 4 |
| | | *Rothia* | sp. | 1 |
| | *Actinomycetaceae* | *Actinomyces* | *oris*[a] | 1 |
| *Proteobacteria* | *Moraxellaceae* | *Acinetobacter* | sp. | 2 |
| | | | *johnsonii*[a] | 1 |
| | *Pseudomonadaceae* | *Pseudomonas* | sp. | 1 |
| | *Comamonadaceae* | *Comamonas* | sp. | 1 |
| | | | *testosteroni* | 1 |
| | | *Acidovorax* | sp. | 1 |
| | *Neisseriaceae* | *Neisseria* | *perflava* | 1 |
| | *Burkholderiaceae* | *Cupriavidus* | sp. | 1 |
| | *Sphingomonadaceae* | *Sphingobium* | sp. | 7 |
| | | | *xenophagum* | 1 |
| | *Enterobacteriaceae* | *Leclercia* | sp. | 1 |
| | | *Enterobacter* | *hormaechei*[a] | 1 |
| | *Oxalobacteraceae* | *Massilia* | *timonae* | 1 |
| | *Caulobacteraceae* | *Brevundimonas* | sp. | 1 |

[a]Species categorized as risk group 2 (BAuA 2015).

pathogenic bacteria. In total, 21 of the 39 identified isolates (54%) were classified as RG 2.

## CONCLUSIONS AND OUTLOOK

Our study revealed a moderate bacterial contamination of holy water samples and a frequent occurrence of potentially pathogenic bacteria, thereby corroborating previous studies. Holy water clearly represents a potential source of infection, in particular for elderly and/or ill and immunocompromised people. Although our study was not performed according to the standard methods of the German Drinking Water Ordinance (Federal Ministry of Justice and Consumer Protection (BMJV) 2001) and ISO 6222 (ISO 1999), our data also suggest that used holy water (as accessible in a church) cannot be recommended for drinking. Admittedly, the applied cultivation conditions might have selected for human-associated bacteria

and discriminated typical (oligotrophic) water bacteria. Nevertheless, our data suggest that human (skin) associated bacteria represent a significant fraction of the holy water microbiota, which (therefore) is also influenced by the number of visitors to a church. In conclusion, holy water in more frequented churches should be replaced more often.

In order to better understand the hygienic relevance of the holy water microbiota and the influence of environmental factors (frequency of use, salt concentration, pH, font building material, etc.), further studies with larger sample sizes and ideally using cultivation-independent techniques should address an extended spectrum of microorganisms including anaerobic bacteria, fungi and also viruses. In addition, appropriate antimicrobial strategies in compliance with religious principles should be investigated in more detail to control microbial growth in holy water fonts and the respective storage containers.

## ACKNOWLEDGEMENTS

The authors cordially wish to thank the local church communities for allowing access to the holy water fonts for microbiological analyses and for providing the congregant numbers of the investigated churches. Furtwangen University is thanked for funding this study. The first two authors contributed equally to this work.

## REFERENCES

BAuA – German Federal Institute for Occupational Safety and Health 2015 Technical Rule for biological agents #446 – Classification of prokaryotes (bacteria and archaea) into risk groups. Edition: August 2015.

BMJV – Federal Ministry of Justice and Consumer Protection 2001 Drinking Water Ordinance. Edition: March 2016.

Daschner, F. D. 1997 Holy water, tap water, mineral water or water filters? *Journal of Hospital Infection* **35**, 71–72.

Egert, M. & Simmering, R. 2015 The microbiota of the human skin. *Advances in Experimental Medicine and Biology* **902**, 61–81.

Fierer, N., Hamady, M., Lauber, C. L. & Knight, R. 2008 The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proceedings of the National Academy of Sciences U.S.A.* **105**, 17994–17999.

Götz, F., Bannerman, T. & Schleifer, K.-H. 2006 The genera staphylococcus and macrococcus. *Prokaryotes* **4**, 5–75.

Greaves, I. & Porter, K. M. 1992 Holy spirit? An unusual case of pseudomonal infection in a multiply injured patient. *British Medical Journal* **305**, 1578.

ISO (International Organization for Standardization) 1999 Enumeration of culturable microorganisms. Colony count by inoculation in a nutrient agar culture medium, ISO 6222.

Jurado, V., Ortiz-Martinez, A., Gonzalez-del Valle, M., Hermosin, B. & Saiz-Jimenez, C. 2002 Holy water fonts are reservoirs of pathogenic bacteria. *Environmental Microbiology* **4**, 612–620.

Kirschner, A., Atteneder, M., Schmidhuber, A., Knetsch, S., Farnleitner, A. & Sommer, R. 2012 Holy springs and holy water: underestimated sources of illness? *Journal of Water and Health* **10**, 349–357.

Khot, P. D., Couturier, M. R., Wilson, A., Croft, A. & Fisher, M. A. 2012 Optimization of matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis for bacterial identification. *Journal of Clinical Microbiology* **50**, 3845–3852.

Michel, M., Entenmann, A., Wiemann, T., van Baalen, A. & Krause, M. 2013 Holy water associated pulmonary infection with a multiresistant Acinetobacter baumanii in an 11-year-old child. *Klinische Pädiatrie* **225**, 43–44.

Phatthararangrong, N., Chantratong, N. & Jitsurong, S. 1998 Bacteriological quality of holy water from Thai temples in Songkhla Province, Southern Thailand. *Journal of the Medical Association of Thailand* **7**, 547–550.

R Core Team 2016 *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rees, J. C. & Allen, K. 1996 Holy water – a risk factor for hospital-acquired infection. *Journal of Hospital Infection* **32**, 51–55.

First received 1 February 2017; accepted in revised form 31 May 2017. Available online 13 July 2017