



# The Washing Machine as a Reservoir for Transmission of Extended-Spectrum-Beta-Lactamase (CTX-M-15)-Producing *Klebsiella oxytoca* ST201 to Newborns

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**ABSTRACT** During the period from April 2012 to May 2013, 13 newborns (1 to 4 weeks of age) and 1 child in a pediatric hospital ward in Germany were colonized with *Klebsiella oxytoca* producing an extended-spectrum beta-lactamase (ESBL) (CTX-M-15). A microbiological source-tracking analysis with human and environmental samples was carried out to identify the source and transmission pathways of the *K. oxytoca* clone. In addition, different hygienic intervention methods were evaluated. *K. oxytoca* isolates were detected in the detergent drawer and on the rubber door seal of a domestic washer-extractor machine that was used in the same ward to wash laundry for the newborns, as well as in two sinks. These strains were typed using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing. The environmental findings were compared with those for the human strains and the isolates detected on clothing. The results from both techniques showed that the strains were identical (sequence type 201 and PFGE type 00531, a clone specific to this hospital and not previously isolated in Germany), emphasizing the washing machine as a reservoir and fomite for the transmission of these multidrug-resistant bacteria. After the washing machine was taken out of use, no further colonizations were detected during the subsequent 4-year period.

**IMPORTANCE** Washing machines should be further investigated as possible sites for horizontal gene transfer (ESBL genes) and cross-contamination with clinically important Gram-negative strains. Particularly in the health care sector, the knowledge of possible (re-)contamination of laundry (patients' clothes and staff uniforms) with multidrug-resistant Gram-negative bacteria could help to prevent and to control nosocomial infections. This report describes an outbreak with a single strain of a multidrug-resistant bacterium (*Klebsiella oxytoca* sequence type 201) in a neonatal intensive care unit that was terminated only when the washing machine was removed. In addition, the study implies that changes in washing machine design and processing are required to prevent accumulation of residual water where microbial growth can occur and contaminate clothes.

**KEYWORDS** ESBL-producing bacteria, *Klebsiella oxytoca*, ST201, washing machine, laundry, colonization, newborns

**W**ater or wastewater contaminated with facultative pathogenic microorganisms provides a potential reservoir for infections (1–3). The most common bacteria causing health care-associated infections or permanently colonizing reservoirs linked to

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contaminated hospital water in the environments of patients are Gram-negative bacteria, including *Pseudomonas* spp., *Enterobacter* spp., *Serratia* spp., *Stenotrophomonas* spp., and *Klebsiella* spp. (1, 2, 4). Such water reservoirs in health care settings include faucets, sink surfaces, bathtubs, wastewater drainage system drains, sinks, showers, and toilets (4, 5). Persisting and especially multidrug-resistant (MDR) bacteria in those reservoirs pose severe risks, especially for high-risk patients such as severely immunocompromised patients, newborns (1 to 4 weeks of age), and infants (6 to 8 weeks of age) in neonatal intensive care units (ICUs) (4, 6–10). For example, Leitner et al. (11) described an outbreak of 6 infections in patients with hematological malignancies that involved a strain of KPC-2-producing *Klebsiella oxytoca*, which is most commonly linked to contaminated water reservoirs. Identification of the contaminated environmental reservoir in such outbreaks can be challenging, and transmission pathways have been elucidated in only a few clinical studies (6, 12, 13).

Currently, washing machines and clothing are not assessed as potential reservoirs in outbreaks of nosocomial infections, despite evidence that a potential health risk due to contaminated laundry cannot be excluded (14–16). Recently, Rehberg et al. (17) described the presence of antibiotic-resistant bacteria (ARB) and their possible transmission via washing machines.

## RESULTS

**Case description.** Between April 2012 and May 2013, increased rates of colonization with *K. oxytoca* isolates were recorded in a level 1 perinatal center (PNC) and in several wards in the connected children's hospital (90 stationary beds and 20 ICU beds) in western Germany. The colonizations had been noticed after implementation of a standard screening procedure for incoming individuals and/or patients with risk factors (newborns, children, and mothers), as a general control mechanism aimed at reducing the spread of MDR bacteria. Screening included anal swabs (newborns, children, and mothers), vaginal swabs (mothers), and in some cases additional wound swabs (newborns and children).

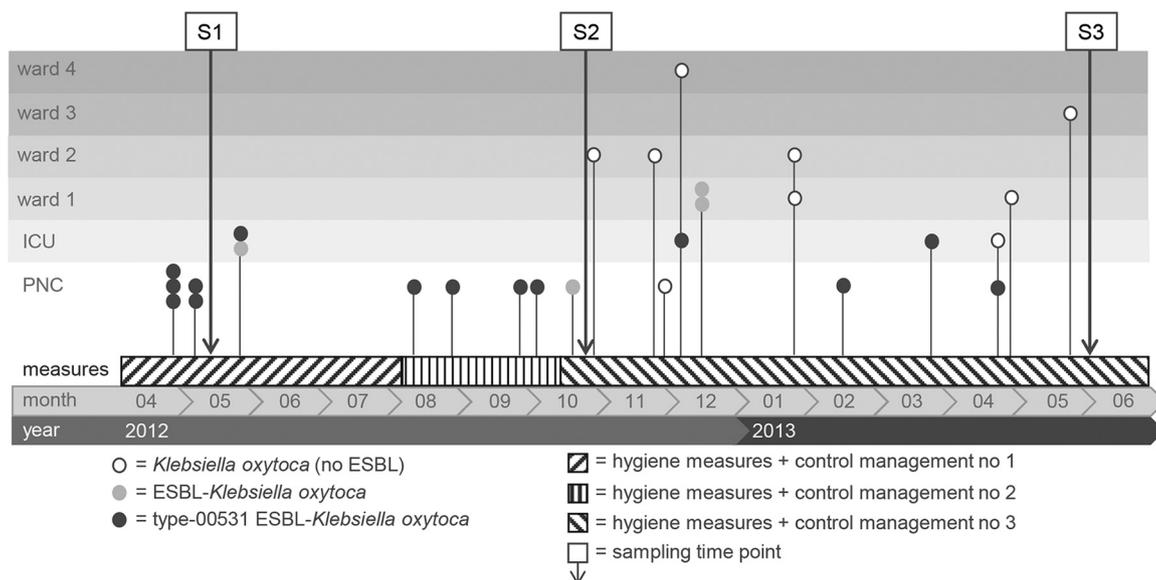
**Colonization with *K. oxytoca* in humans.** Within 14 months, 27 children were colonized (but not infected) with *K. oxytoca*, according to CDC definitions (18). The occurrence of *K. oxytoca* varied from sensitive isolates with no extended-spectrum beta-lactamase (ESBL) activity to ESBL-producing *K. oxytoca* isolates and the particular ESBL-producing *K. oxytoca* pulsed-field gel electrophoresis (PFGE) type 00531. Fourteen children tested positive for PFGE type 00531 ESBL-producing *K. oxytoca*.

*K. oxytoca* was mostly identified in rectal swab screening samples. For 9 of 24 newborns, *K. oxytoca* was also detected in throat swab samples. As an exception, a 4-year-old boy in the ICU who never had any direct contact with the PNC also showed rectal colonization with PFGE type 00531/sequence type 201 (ST201) ESBL-producing *K. oxytoca* in March 2013.

All ESBL-producing *K. oxytoca* strains were resistant to piperacillin-tazobactam (MICs up to and higher than the measurable limit of 64/4 mg/liter), cefotaxime (MICs of  $\geq 64$  mg/liter), ceftazidime (MICs of  $>128$  mg/liter), and ciprofloxacin (MICs of  $\geq 2$  mg/liter) and could be classified as MDR according to the guidelines reported by Magiorakos et al. (19). Figure 1 summarizes the occurrence of *K. oxytoca* isolates over time in the PNC, the ICU, and four different wards.

Until October 2012, only newborns in the PNC or infants in the ICU were colonized with ESBL-producing *K. oxytoca*, some strains of which belonged to PFGE type 00531/ST201 (Fig. 1). From the end of October 2012 to February 2013, *K. oxytoca* was also detected on other wards and among older pediatric patients (see above).

During an extended screening of mothers and health care workers (HCWs), a total of 695 swabs (vaginal and rectal) from 428 persons were obtained on the obstetrics ward. The screening identified 4 mothers colonized with *K. oxytoca*, 5 mothers colonized with ESBL-producing *Escherichia coli*, and 1 mother colonized with ESBL-producing *Klebsiella pneumoniae*. None was positive for PFGE type 00531 ESBL-



**FIG 1** Course of the outbreak with different *K. oxytoca* strains over 1 year and their distributions in different wards. S1, first sampling; S2, second sampling; S3, third sampling.

producing *K. oxytoca*. Transmission between a mother and a newborn was not documented.

**Environmental monitoring.** All environmental samples obtained during the first and second on-site inspections tested negative for *Enterobacteriales* and nonfermenting organisms. During the second on-site sampling, in October 2012, only low concentrations of Gram-positive skin and environmental bacteria were present. During the third on-site inspection, in June 2013, sampling was conducted using native samples (drinking water and wastewater) and liquid medium swabs (ESwabs). Thus, *Enterobacteriales* and nonfermenting bacteria were detected. All *K. oxytoca* isolates were identical ESBL-producing strains and belonged to PFGE type 00531 (Table 1). In the following discussion, these *K. oxytoca* strains are termed environmental.

Water-associated bacteria, such as *Pseudomonas aeruginosa*, *Serratia* spp., *Enterobacter* spp., *K. pneumoniae*, and *Stenotrophomonas maltophilia*, were detected in the siphons of hand wash basins. Identical clones of PFGE type 00531/ST201 *K. oxytoca* were isolated from the siphons of two sinks in the HCW staff room and in the room used for cleaning and disinfection. The same clone was also isolated at high concentrations from samples of residual water in the rubber seal and from a swab sample (in addition to *P. aeruginosa*) from the detergent compartment of one of the two washing machines (Table 1) located on the ground floor of the same ward.

Following identification of the washing machine as a potential reservoir for this specific *K. oxytoca* clone, newborn clothing (hats and socks) that had been washed in this machine were microbiologically analyzed. PFGE type 00531/ST201 *K. oxytoca* of the same specific clone was isolated, with total counts of >10<sup>9</sup> CFU/ml. All PFGE type 00531/ST201 ESBL-producing *K. oxytoca* isolates found in the residual water and environmental samples were also resistant to piperacillin-tazobactam (MICs ranging from 8/4 mg/liter to >64/4 mg/liter), cefotaxime (MICs of >2 mg/liter), ceftazidime (MICs of 64 mg/liter), and ciprofloxacin (MICs of ≥ 2 mg/liter) (Table 2).

The presence of the CTX-M-15 ESBL gene in all human and environmental strains was confirmed by sequencing. The transfer of putative plasmids harboring these genes under laboratory conditions into wild-type *E. coli* strains via conjugation was not successful. In addition, no amplicons for *inc* genes (*incA/C*, *incL*, *incHI2*, *incM*, *incI1-αγ*, *incN*, *incFIIk*, *incFIA*, *incFIB*, and *incFII*) were obtained from the strains, indicating that the CTX-M genes might be chromosomally located, as described by Rodríguez et al. (20).

**TABLE 1** Occurrence of Gram-negative *Enterobacteriales* strains, nonfermenting organisms, and PFGE type 00531 ESBL-producing *K. oxytoca* isolates in environmental samples obtained during on-site inspection of risk areas

| Room                             | Sampling point                                   | Counts (CFU/ml or total Gram-negative bacterial count) | Microbiological differentiation   | CTX-M                            | PFGE type 00531/ST201 <i>K. oxytoca</i> |
|----------------------------------|--|--|---|----------------------------------|---|
| Nursing care room, storage rooms | Working basin, siphon                            | $2.1 \times 10^5$                                      | <i>Serratia marcescens</i>  | Positive                         | Yes                                     |
|                                  | Hand wash basin, siphon water                    | $1.6 \times 10^6$                                      | <i>K. oxytoca</i><br><i>Raoultella terrigena</i><br><i>Enterobacter cloacae</i> complex | Negative<br>Negative<br>Negative |   |
| Staff toilet                     | Hand wash basin, drainage hole cover             | Scattered/swab (no CFU/ml)                             | <i>E. cloacae</i> complex<br><i>K. pneumoniae</i>                                       | Negative<br>Negative             |   |
|                                  | Hand wash basin, fresh water                     | Not evaluable/100 ml                                   | Gram-negative nonfermenting rods  |                                  |   |
|                                  | Hand wash basin, siphon water<br>Toilet, water   | $1.5 \times 10^4$<br>1.0                               | <i>P. aeruginosa</i> , <i>S. maltophilia</i><br><i>S. maltophilia</i>                   |                                  |   |
| Staff room                       | Kitchen sink, siphon water                       | $3.0 \times 10^5$                                      | <i>K. oxytoca</i><br><i>P. aeruginosa</i><br><i>S. maltophilia</i>                      | Positive<br>Negative             | Yes                                     |
|                                  | Water reservoir tank, espresso machine           | 10.0 CFU/50 ml   |   |                                  |   |
| Hygiene sluice, PNC              | Hand wash basin, siphon water                    | $1.3 \times 10^4$                                      | <i>P. aeruginosa</i> , Gram-negative nonfermenting rods                                 |                                  |   |
|                                  | Tumble dryer, water tray                         | Not evaluable/50 ml                                    |   |                                  |   |
| Basement, laundry room           | Washing machine 1, residual water, rubber mantle | $3.9 \times 10^2$                                      | Gram-negative nonfermenting rods  | Positive                         | Yes                                     |
|                                  | Washing machine 1, detergent compartment         | Not evaluable/swab                                     | <i>K. oxytoca</i><br><i>P. aeruginosa</i>   | Positive<br>Negative             | Yes                                     |
|                                  | Washing machine 2, detergent compartment         | Not evaluable/swab                                     |   |                                  |   |
|                                  | Hat 1  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |
| Laundry                          | Hat 2  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |
|                                  | Hat 3  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |
|                                  | Hat 4  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |
|                                  | Socks 1  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |
|                                  | Socks 2  | $>10^9$  | <i>K. oxytoca</i>   | Positive                         | Yes                                     |

**TABLE 2** MICs of *K. oxytoca* isolates obtained during on-site inspection of risk areas

| No. | Species           | Sample type                           | MIC (mg/liter) <sup>a</sup> |                         |            |             |          |            |          |               |
|-----|-------------------|---------------------------------------|-----------------------------|-------------------------|------------|-------------|----------|------------|----------|---------------|
|     |                   |                                       | Piperacillin                | Piperacillin-tazobactam | Cefotaxime | Ceftazidime | Imipenem | Meropenem  | Amikacin | Ciprofloxacin |
| 1   | <i>K. oxytoca</i> | Washing machine                       | R (>16)                     | I (16/4)                | R (>2)     | R (32)      | S (≤1)   | S (≤0.125) | S (≤4)   | R (2)         |
| 2   | <i>K. oxytoca</i> | Washing machine                       | R (>16)                     | R (>64/4)               | R (>2)     | R (32)      | S (≤1)   | S (≤0.125) | S (8)    | R (2)         |
| 3   | <i>K. oxytoca</i> | Washing machine                       | R (>16)                     | R (64/4)                | R (>2)     | R (32)      | S (≤1)   | S (≤0.125) | S (8)    | R (2)         |
| 4   | <i>K. oxytoca</i> | Siphon from wash basin in staff lobby | R (>16)                     | R (32/4)                | R (>2)     | R (32)      | S (≤1)   | S (≤0.125) | S (8)    | R (>2)        |
| 5   | <i>K. oxytoca</i> | Siphon from wash basin in staff lobby | R (>16)                     | S (8/4)                 | R (>2)     | R (32)      | S (≤1)   | S (≤0.125) | S (≤4)   | R (2)         |
| 6   | <i>K. oxytoca</i> | Siphon from wash basin in staff lobby | R (>16)                     | I (16/4)                | R (>2)     | R (64)      | S (≤1)   | S (≤0.125) | S (≤4)   | R (2)         |
| 7   | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | I (16)   | R (>2)        |
| 8   | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (8)    | R (>2)        |
| 9   | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (8)    | R (>2)        |
| 10  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (≤4)   | R (>2)        |
| 11  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (8)    | R (>2)        |
| 12  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (≤4)   | R (>2)        |
| 13  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (≤4)   | R (2)         |
| 14  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (≤4)   | R (>2)        |
| 15  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (8)    | R (>2)        |
| 16  | <i>K. oxytoca</i> | Human                                 | R (>16)                     | R (>64/4)               | R (>2)     | R (>128)    | S (≤1)   | S (≤0.125) | S (≤4)   | R (>2)        |

<sup>a</sup>R, resistant; I, intermediate; S, sensitive.

**Epidemiological links.** Retrospective analysis demonstrated that only newborns who had worn clothing that had been washed in the in-house washing machine were colonized with the redundant *K. oxytoca* clone. Although the siphons of the staff sinks were also identified as a possible reservoir, no staff members were identified as carriers or spreaders of ESBL-producing *Enterobacteriales* at the time of screening.

All clinical and environmental isolates of PFGE type 00531/ST201 *K. oxytoca* displayed identical PFGE banding patterns and thus were considered clonally identical. This clone was specific for the newborns/infants and some environmental samples.

**Evaluation of possibly increased disinfectant tolerance of the *K. oxytoca* strains.** The environmental *K. oxytoca* isolates showed no increased tolerance to the surface disinfectant based on quaternary ammonium compounds and alkylamine. The alkylamine MICs for the *K. oxytoca* strains were identified as up to a maximum of 0.003%, compared to 0.0075% for the *Pseudomonas aeruginosa* reference strain. Furthermore, the examination according to Verbund für Angewandte Hygiene e.V. (VAH) method 9 showed reductions in the *K. oxytoca* strains of  $\geq 5 \log_{10}$  CFU at a concentration of 0.5% for 15 min in the presence of interfering substances (3.0 g/liter bovine serum albumin plus 3.0 ml/liter sheep erythrocytes [i.e., dirty conditions]). The VAH-recommended concentration and application time are 0.5% for 60 min (<https://vah-online.de/en/for-users>).

*K. oxytoca* isolates from the environment showed no increased tolerance to the laundry disinfectant based on peracetic acid. The MICs of the *K. oxytoca* strains and the *P. aeruginosa* reference strain were identified as 5 g/liter. The analysis according to VAH method 9 (in the presence of 2.75 g/liter laundry disinfectant with an exposure time of 15 min, under dirty conditions, at 20°C, 40°C, and 60°C) demonstrated that the *K. oxytoca* strains were more susceptible than the Gram-negative reference strain *P. aeruginosa* DSM 939. At a temperature of 60°C, all *K. oxytoca* strains showed reductions of  $\geq 5 \log_{10}$  units.

**Hygienic containment measures and control management strategies.** A task force was formed to manage the control interventions for prevention of the colonization of additional patients and to identify possible sources of the suspected *K. oxytoca* strain. The management procedures were divided into three main phases, until the colonizations were under control and the source could finally be identified. In each phase, step-by-step approaches with different strategies were necessary, in parallel with sampling during the whole reporting period (Fig. 1 and Table 3).

**TABLE 3** Hygienic measures and control management procedures

| Time period               | Procedure(s)   |
|---------------------------|--|
| April to August 2012      | Environmental monitoring, including sampling of siphons, sinks, and showers via contact plates<br>Continuation of admission screening and weekly routine screening and implementation of second newborn screening (48 h postpartum)<br>Screening of all mothers at admission and at discharge<br>Monitoring of pathways of newborns from birth to admission in the PNC<br>Maintenance of incubator isolation until two-time negative screening results<br>Advanced training of HCWs, with special focus on toilet and hand hygiene<br>Microbiological analyses of vaginal ultrasound probes and heated towels used for primary care of newborns<br>Renovation/decontamination of ward rooms and removal of unused sinks<br>Installation of new wall-mounted disinfection dispensers<br>Screening of clinical procedures for any cross-contamination possibilities<br>Feeding of newborns with only precooked single-serving packages<br>Use of sterile water for bathing of newborns |
| August to October 2012    | Screening of all HCWs working in the PNC, ICU, or obstetrics department  |
| October 2012 to June 2013 | Extended environmental sampling from different areas in the ward and functionally linked areas in the hospital, with identification and sampling of specific risk factors (e.g., washing machine)<br>Training of staff in correct handling of disinfectant wipes<br>Training of HCWs in correct disinfection and cleaning of incubators<br>Preparation of disinfectant wipes with newly validated/approved preparation machine<br>Construction measures to provide work space for hygienic processing of incubators and to ensure sufficient storage capacities<br>Marking of all thermometers (rectal and commercially available thermometers) and incubators for traceability<br>Intensive audit of all procedures used in the ward during routine operations  |

#### Long-term clinical effects and concomitant infection control interventions.

After the washing machine had been taken out of use, no further colonization of newborns with *K. oxytoca* has been detected to this day. All garments worn by newborns and children were laundered by a professional external hospital laundry service after the outbreak. The two colonized sinks were replaced by sinks with specialized thermosiphon systems. As a further consequence of the prolonged cluster process, the existing infection control measures (isolating colonized patients, enforcing hand hygiene measures, and cleaning the ward, particularly the sinks and equipment) were reinforced via extra training. The screening plan for all newborns and children and all measures implemented within the containment program continue to be maintained.

## DISCUSSION

**Reservoirs and transmission routes for waterborne pathogens in health care systems.** When the first 5 cases of colonization with *K. oxytoca* were detected, between August and October 2012, person-to-person transmission was suggested. Although this has not been reported for *K. oxytoca*, Price et al. (21) showed that, in the presence of standard infection control measures, HCWs were frequent sources of *Staphylococcus aureus* transmission. Furthermore, Heudorf et al. (22) mentioned that pathogens classified by the World Health Organization as highest priority can be found on staff gowns, which can be the starting point for transmissions (23).

In this case, the occurrence of *K. oxytoca* isolates continued over a 1-year period, which indicated a reservoir in the environment rather than among hospital personnel or mothers. Despite the strict implementation of control management and containment measures, newborns and children continued to acquire the cluster organism (PFGE type 00531/ST201), which had never been detected previously by the German National Reference Centre (GNRC). The single database strain was isolated in the Netherlands in 2018 (24).

**Washing machine.** In this case, the particular *K. oxytoca* strain was detected not only in two sinks but also in a domestic washing machine with an integrated dryer. Even recently published articles reviewing the main water-associated reservoirs in hospitals do not consider washing machines as potential hazards in clinical environ-

ments (4, 5). In our case, however, the knitted clothes of patients were washed in an in-house washing machine designed for household use and enabling a washing temperature of 65°C. It has been shown that resistance genes, as well as different microorganisms, can persist in domestic washing machines (17, 25).

Recently, a study suggested the potential role of washing machines in the distribution of antibiotic-resistant Gram-negative bacteria during laundering (17); until now, however, no transmission of pathogens from a washing machine to patients could be proven. In this case, we assume that the PFGE type 00531/ST201 *K. oxytoca* strain was disseminated to clothing after the washing process, via residual water on the rubber mantle and/or via the final rinsing process, which ran unheated and detergent-free water through the detergent compartment, although the water coming to the washing machine met the quality requirements of the hospital's internal water controls. Consequently, we concluded that newborns were colonized by wearing hats and socks that had been contaminated by the washing process.

It remains unclear how the washing machine was contaminated. However, *Enterobacteriales* species can survive in wet environments for extended periods (1), especially waterborne bacteria such as *P. aeruginosa* and *Klebsiella* strains, which have the ability to survive in a viable but nonculturable state. Their environmental stability is supported by the formation of biofilms to enhance their chances of multiplication and horizontal gene transfer (26). Zhang et al. (27) reported that biofilm detachment is promoted by disinfectants, thus affecting the overall antibiotic resistance of microbes in tap water.

Furthermore, Rehberg et al. (17) demonstrated that ARB can survive the washing process. In tests with *P. aeruginosa* outbreak strains, even the use of temperatures above 50°C could not achieve secure reduction of those strains. Although a 65°C washing program had been used for every wash cycle, it is likely that the temperature in the area of the rubber mantle or the rubber door seal would have been much lower, providing an optimal humid environment and nutrient supply for the growth of Gram-negative microorganisms (26, 28). Moreover, the occasional use of washing machines at low temperatures supports the formation of biofilms (14).

It is important to emphasize that the use of regular domestic washing machines for washing patients' clothing in a clinical setting is not permitted, according to the current hygiene regulations in Germany. However, the washing machine concerned was located outside the actual central laundry preparation area and was intended only for washing mothers' clothes and, as in this case, for washing the caps and socks; it was operated exclusively by the nursing staff.

**Conclusion.** While previous studies implicated sinks as potential reservoirs for clusters of infection caused by *K. oxytoca*, this report focuses on the washing machine. The extensive period of the outbreak shows that, with several potential environmental reservoirs, a multidimensional containment approach is necessary. Here, the approach included reinforcement of infection control policies, such as hand hygiene, contact precautions, isolation, admission/routine rectal screening, and clear delineation between handwashing sinks and sinks used for other purposes. However, the ongoing colonization was terminated only after the washing machine was removed and only disposable caps and socks were used. The results suggest that, for prompt management of outbreaks or colonization clusters, the choice of environmental sampling points and the use of effective methods should take into account the ecological properties of the causative strain. Indeed, the environmental strain was not found before the third on-site inspection, as the use of ESswabs allowed better detection.

In addition, the study implies that changes in washing machine design and processing are required to prevent the accumulation of residual water where microbial growth can occur and contaminate clothes. Furthermore, the use of professional washing machines and routine checking with a temperature logger are urgent requirements. In summary, the present study shows that, in situations where an increase in colonization is observed, nonprofessional washing machines, if used, and clothes

should be assessed and investigated as potential reservoirs and vectors for transmission.

## MATERIALS AND METHODS

**Investigation strategy.** When the first colonizations were detected in April 2012, an initial management strategy to analyze the cases and to identify possible sources of the *K. oxytoca* strain was developed. Three major sampling events for all newborns, children, and mothers in the distinct wards took place during the reported time period, following a standard screening procedure.

In March 2013, sampling of specific risk areas (water/wastewater reservoirs) was performed, and ESBL screening (anal swabs and wound swabs) of all HCWs (physicians, nurses, and cleaning personal working in the PNC, the ICU, and the obstetrics department) was conducted to identify possible carriers. In June 2013, further sampling was conducted, using native samples (drinking water and wastewater) and liquid medium swabs (ESwabs).

**Laboratory analyses. (i) Microbiological cultivation.** Human and environmental samples were collected, stored at 4°C during transport to the laboratories, and processed within 48 h. All human samples (anal, vaginal, and wound swabs) were streaked on MacConkey agar plates (Oxoid Deutschland GmbH, Wesel, Germany) with a 10- $\mu$ g imipenem disk (bioMérieux SA, Marcy-Étoile, France) and selective agar plates, e.g., CHROMagar ESBL plates (Oxoid Deutschland GmbH). Oxoid Rodac contact plates (diameter of 55 mm; Becton, Dickinson, Heidelberg, Germany) were used for environmental surface sampling and were incubated at 36°C  $\pm$  1°C for 48 h. All environmental liquid medium swabs (ESwab; Copan Diagnostics, Italy) were streaked on Columbia agar plates containing 5% sheep blood (Becton, Dickinson), MacConkey agar plates (product no. PO5002A; Oxoid Deutschland GmbH), and selective agar plates (e.g., CHROMagar ESBL plates, product no. 43481; bioMérieux), and the plates were incubated at 37°C  $\pm$  1°C for 48 h. In addition, casein soy flour peptone broth (CASO broth) (Merck, Darmstadt, Germany) was inoculated with the same swabs and incubated at 37°C  $\pm$  1°C for 24 h.

The disinfectant wipes, socks, and knitted hats of the newborns were put into 200 ml of CASO broth with Tween-saponin-histidine-cysteine (TSHC) (3% Tween [product no. 8.22187; Merck], 3% saponin [product no. 5185.1; Roth], 0.1% histidine [product no. 1.04351; Merck], 0.1% cysteine [product no. 1.02839; Merck]) and homogenized for 60 s (Stomacher 400 blender; Seward Ltd., West Sussex, United Kingdom). Two 100-ml aliquots of the extract were filtered (Millipore Hydrosol membrane filter, with 250-ml cup), followed by the filtration of 100 ml of 0.9% NaCl to rinse the additives off the filter. The filter was then placed on CHROMagar ESBL and MacConkey agar plates, and the plates were incubated at 37°C for 24 to 48 h. In addition, 100  $\mu$ l of each concentration of a dilution series in 0.9% NaCl was plated on CHROMagar ESBL and MacConkey agar plates. The emulsions were treated in the same manner as the wipes, except that shredding was not necessary.

For the water samples, approximately 100 ml was collected in a sterile polystyrene cup (product no. 225170; Greiner, Frickenhausen, Germany) and filtered through a sterile nitrocellulose membrane filter (pore size of 0.45  $\pm$  0.02  $\mu$ m and diameter of 47 mm, with a black grid, product no. EZHAWG 474; Millipore), according to the method described by Schulz and Hartung (29). After filtration, the membrane was placed on selective CHROMagar ESBL and MacConkey agar plates. In addition, 100  $\mu$ l of each dilution of a dilution series in 0.9% NaCl was plated on CHROMagar ESBL and MacConkey agar plates.

**(ii) Identification and susceptibility testing of ARB.** All *Enterobacteriales* strains detected on CHROMagar ESBL plates were identified by an API 20E system with Apiweb v4.1 (bioMérieux) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (bioMérieux) using Myla software, the Vitek MS- $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) matrix (product no. 411 071; bioMérieux), and disposable targets (product no. 410 893; bioMérieux). Susceptibility to antibiotics was tested using the Micronaut-5 MDR MRGN-Screening 3 system (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany), and results were interpreted using EUCAST criteria.

**(iii) Testing of disinfectant tolerance.** To detect possibly increased tolerance of the *K. oxytoca* isolates to disinfectants, surface disinfectant and the disinfecting general-purpose detergent that had been used in the washing machines were both examined according to the regulations (methods 7 and 9) of the Disinfectant Commission of the VAH (30). VAH method 7 was used to determine the MICs of the environmental *K. oxytoca* strains, in comparison with a Gram-negative reference strain, *Pseudomonas aeruginosa* DSM 939 (31). VAH method 9 is a quantitative suspension test that was used to identify efficacy gaps, taking into account the concentration-time relationship, interfering substances, and temperature of the disinfectants (32–34). The surface disinfectant used was based on quaternary ammonium compounds and alkylamine [didecyldimethylammoniumchloride, 12.5 g/100 g; bis-(aminopropyl)laurylamine, 1.5 g/100 g]. The laundry disinfectant was based on peracetic acid (40 to 80 ppm peracetic acid, 2.5 to 5 g/liter).

**Molecular resistance characterization. (i) Molecular characterization of ESBL genes.** Possible ESBL production was confirmed by PCR. Five specific primer sets were used to detect beta-lactamase-encoding genes belonging to the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub> families (35–38). The PCR products were visualized by gel electrophoresis on a 1% agarose-Tris-borate-EDTA (TBE) gel and stained with Midori green (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The resulting amplicons were purified using an innuPREP DOUBLEpure kit (Analytik Jena AG, Jena, Germany), according to the manufacturer's recommendations. Custom sequencing was performed by Microsynth (Göttingen, Germany). The nucleotide sequences were analyzed using Chromas 2.6.5.

**(ii) Plasmid characterization (Inc typing).** To distinguish the Inc types of possible plasmids, 2 or 3 colonies of fresh cultures of all *K. oxytoca* isolates were resuspended in 100  $\mu$ l of nuclease-free water, heated to 95°C for 10 min, and centrifuged at 14,000  $\times$  *g* for 5 min. The supernatant was carefully

**TABLE 4** Primers used for Inc typing

| Primer                     | Sequence                          | Reference             |
|----------------------------|-----------------------------------|-----------------------|
| IncHI2_fw                  | TTT CTC CTG AGT CAC CTG TTA ACA C | Carattoli et al. (40) |
| IncHI2_rev                 | GGC TCA CTA CCG TTG TCA TCC T     | Carattoli et al. (40) |
| IncA/C_fw                  | GAG AAC CAA AGA CAA AGA CCT GGA   | Carattoli et al. (40) |
| IncA/C_rev                 | ACG ACA AAC CTG AAT TGC CTC CTT   | Carattoli et al. (40) |
| IncL_fw                    | CGG AAC CGA CAT GTG CCT ACT       | Carattoli et al. (41) |
| IncM_fw                    | GGA TGA AAA CTA TCA GCA TCT GAA G | Carattoli et al. (41) |
| IncL/M_rev                 | GAA CTC CGG CGA AAG ACC TTC       | Carattoli et al. (41) |
| IncI1- $\alpha\gamma$ _fw  | CGA AAG CCG GAC GGC AGA A         | Carattoli et al. (40) |
| IncI1- $\alpha\gamma$ _rev | TCG TCG TTC CGC CAA GTT CGT       | Carattoli et al. (40) |
| IncN_fw                    | GTC TAA CGA GCT TAC CGA AG        | Carattoli et al. (40) |
| IncN_rev                   | GTT TCA ACT CTG CCA AGT TC        | Carattoli et al. (40) |
| IncFIIIk_fw                | TCT TCT TCA ATC TTG GCG GA        | Villa et al. (39)     |
| IncFIIIk_rev               | GCT TAT GTT GCA CRG AAG GA        | Villa et al. (39)     |
| IncFIA_fw                  | CCA TGC TGG TTC TAG AGA AGG TG    | Carattoli et al. (40) |
| IncFIA_rev                 | GTA TAT CCT TAC TGG CTT CCG CAG   | Carattoli et al. (40) |
| IncFIB_fw                  | TCT GTT TAT TCT TTT ACT GTC CAC   | Villa et al. (39)     |
| IncFIB_rev                 | CTC CCG TCG CTT CAG GGC ATT       | Villa et al. (39)     |
| IncFII_fw                  | CTG ATC GTT TAA GGA ATT TT        | Villa et al. (39)     |
| IncFII_rev                 | CAC ACC ATC CTG CAC TTA           | Villa et al. (39)     |

removed and used for PCR. The PCR mixture contained 2× OneTaq master mix (New England Biolabs) and 0.2  $\mu$ M each primer (Table 4) (39–41).

The PCR conditions were set at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. The final extension was performed at 68°C for 5 min. The PCR products were analyzed using agarose gel electrophoresis on a 1% agarose-TBE gel and were stained with Midori green (Biozym Scientific GmbH).

**(iii) Plasmid conjugation testing.** The possibility of plasmid transfer via conjugation was analyzed. All *K. oxytoca* and wild-type *E. coli* strains were streaked onto LB agar containing (i) 20 mg/liter chloramphenicol or (ii) 20 mg/liter chloramphenicol and 4 mg/liter cefotaxime. *E. coli* strains that grew on LB agar containing only chloramphenicol but not on LB agar containing both chloramphenicol and cefotaxime were used for the following conjugation assay. For the preculture, 1 colony of fresh cultures of all isolated *K. oxytoca* strains (donors) and different wild-type *E. coli* strains (recipients) were used for inoculation of 5 ml lysogeny broth, and the broth was incubated overnight at 37°C, with shaking. Next, 5 ml of fresh lysogeny broth was inoculated with 100  $\mu$ l of the donor (*K. oxytoca*) and 1 ml of the recipient (*E. coli*) and incubated overnight at 37°C, with shaking. After incubation, 1 ml of each culture was centrifuged at 14,000 rpm for 3 min. Subsequently, the supernatant was discarded and the pellet was resuspended using 100  $\mu$ l of Milli-Q water. The whole suspensions, as well as 100  $\mu$ l of the cultures, were plated onto selective LB agar containing 20 mg/liter chloramphenicol and 4 mg/liter cefotaxime and were incubated overnight at 37°C. Grown colonies were streaked onto Columbia sheep blood agar plates and analyzed via MALDI-TOF MS.

**(iv) Molecular typing.** All *K. oxytoca* strains isolated from clinical samples and environmental sources were typed by the GNRC using PFGE, according to the method described by Tenover et al. (42) and modified as described by Ribot et al. (43). In addition, multilocus sequence typing (MLST) of all *K. oxytoca* strains was conducted. Briefly, bacteria were cultivated overnight at 35°C on Columbia blood agar plates. After resuspension of 2 or 3 colonies in 80  $\mu$ l of PCR water, the samples were heated to 95°C for 5 min and centrifuged at 14,000  $\times$  g for 5 min. The supernatant was used for PCR according to the protocol described by Herzog et al. (44). The PCR products were purified by extraction from a 1% agarose gel using the GeneJET gel extraction kit (Fisher Scientific GmbH, Schwerte, Germany), according to the manufacturer's recommendations, and were sequenced by GATC Biotech AG (Constance, Germany). Assignment to the ST was performed via the specific MLST website (<https://pubmlst.org/koxytoca>).

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