

1           **The washing machine as a reservoir for transmission of**  
2           **extended spectrum beta-lactamase (CTX-M-15)-producing**  
3           ***Klebsiella oxytoca* ST201 in newborns**

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5 Ricarda M. Schmithausen<sup>a#</sup>, Esther Sib<sup>a</sup>, Martin Exner<sup>a</sup>, Sylvia Hack<sup>a</sup>, Claudia Rösing<sup>a</sup>,  
6 Patrick Ciorba<sup>a</sup>, Gabriele Bierbaum<sup>b</sup>, Mykhailo Savin<sup>c</sup>, Sally F. Bloomfield<sup>d</sup>, Martin Kaase<sup>e</sup>,  
7 Anja Jacobshagen<sup>a</sup>, Stefanie Gemein<sup>a</sup>, Jürgen Gebel<sup>a</sup>, Steffen Engelhart<sup>a</sup>, Daniel Exner<sup>f</sup>

8  
9 <sup>a</sup>Institute for Hygiene and Public Health, University Hospital Bonn, Venusberg-Campus 1,  
10 53127 Bonn, Germany

11 <sup>b</sup>Institute of Medical Microbiology, Immunology and Parasitology, Venusberg-Campus 1,  
12 53127 Bonn, Germany

13 <sup>c</sup>Institute of Animal Science, Department of Preventive Health Management,  
14 Katzenburgweg 7-9, 53115 Bonn, Germany

15 <sup>d</sup>London School of Hygiene and Tropical Medicine, University of London, Keppel St,  
16 London WC1E 7HT, United Kingdom

17 <sup>e</sup>National Reference Laboratory for multidrug-resistant gram-negative bacteria,  
18 Department of Medical Microbiology, Ruhr-University Bochum, Germany. Current  
19 address: Department for Infection Control and Infectious Diseases, University Medical  
20 Center Göttingen, Kreuzberggring 57, 37075 Göttingen, Germany

21 <sup>f</sup>Department of General, Visceral, Thoracic and Vascular Surgery, University Hospital  
22 Bonn, Venusberg-Campus 1, 53127 Bonn, Germany

23

24 Running Head: The washing machine as a reservoir for ESBL-bacteria

25

26 # Address correspondence to Ricarda Schmithausen, ricarda.schmithausen@ukbonn.de

27

28

29 **Abstract**

30 During the time period from April 2012 to May 2013, 13 newborns (1 – 4 weeks old) and  
31 one child in a paediatric hospital ward in Germany were colonized with an extended  
32 spectrum betalactamase (ESBL) producing *Klebsiella oxytoca* (CTX-M-15).

33 A microbiological source-tracking analysis, including human and environmental samples,  
34 was carried out to identify the source and transmission pathways of the *K. oxytoca* clone.  
35 In addition, different hygienic intervention methods were evaluated.

36 Isolates of the *K. oxytoca* strains were detected in the detergent drawer and on the rubber  
37 door seal of a domestic washer-extractor machine, used in the same ward to wash laundry  
38 of the newborns as well as in two sinks. These strains were typed using PFGE and multi-  
39 locus sequence typing (MLST). The environmental findings were compared with those of  
40 the human strains and the isolates detected on the clothing. The results showed that the  
41 strains were identical using both techniques, (ST201 and pulse-field-gel-electrophoresis  
42 [PFGE] type-00531, a clone specific to this hospital and not previously isolated in  
43 Germany), emphasizing the washing machine as a reservoir and fomite for the  
44 transmission of these multidrug-resistant bacteria (MDR). After the washing machine had  
45 been taken out of use, no further colonizations were detected over a following 4-year  
46 period.

47

48 **Importance**

49 Washing machines should be further investigated as possible sites for horizontal gene  
50 transfer (ESBL-genes) and cross-contamination of clinically important Gram-negative  
51 strains. Particularly in the healthcare sector, the knowledge of possible (re-) contamination  
52 of laundry (patients' clothes, staff uniforms) with multidrug-resistant Gram-negative

53 bacteria could help to prevent and control nosocomial infections. This report describes an  
54 outbreak with a single strain of a multidrug-resistant bacterium (*Klebsiella oxytoca* ST201)  
55 in a neonatal intensive care unit, that was only terminated when the washing machine was  
56 removed. In addition, the study implies that changes in washing machine design and  
57 processing are required to prevent accumulation of residual water where microbial growth  
58 can occur and contaminate clothes.

59

60 **Keywords**

61 ESBL-bacteria, *Klebsiella oxytoca*, ST201, washing machine, laundry, colonization,  
62 newborns

63

64 **Introduction**

65 Water or wastewater contaminated with facultative pathogenic microorganisms provides  
66 a potential reservoir for infections (1-3). The most common bacteria causing healthcare-  
67 associated infections or permanently colonizing reservoirs linked to contaminated hospital  
68 water in the environment of patients are Gram-negative bacteria including *Pseudomonas*  
69 spp., *Enterobacter* spp., *Serratia* spp., *Stenotrophomonas* spp. and *Klebsiella* spp. (1, 2,  
70 4). Such water reservoirs in healthcare settings include faucets, sink surfaces, bathtubs,  
71 wastewater drainage system drains, sinks, showers, and toilets (4, 5). Persisting and  
72 especially multidrug-resistant bacteria in those reservoirs pose a severe risk especially for  
73 high-risk patients, including severely immunocompromised patients, newborns (1-4 weeks  
74 old) and infants (6–8 weeks old) in neonatal intensive care units (ICU) (4, 6-10). For  
75 example, Leitner et al. (11) described an outbreak of six infections in patients with  
76 hematological malignancies with a KPC-2-producing *K. oxytoca* most commonly linked to  
77 contaminated water reservoirs. In these outbreaks, the identification of the contaminated  
78 environmental reservoir can be challenging and transmission pathways have been  
79 elucidated in only a few clinical studies (6, 12, 13).

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

85

86 **Case description**

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

95

## 96 **Material and Methods**

### 97 ***Investigation strategy***

98 When the first colonizations had been detected in April 2012, an initial management  
99 strategy to analyse the cases and identify possible sources of the *K. oxytoca* strain was  
100 developed: Three major sampling events of all newborns, children and mothers at the  
101 distinct wards took place during the reported time period, following a standard screening  
102 procedure.

103 In March 2013 sampling of specific risk areas (water/wastewater reservoirs) was  
104 performed and ESBL-screening (anal, wounds) of all healthcare workers (physicians,  
105 nurses, and cleaning personal working in the PNC, the ICU, and the Obstetrics  
106 Department) was conducted to identify possible carriers.

107 In June 2013, a further sampling was conducted, using native samples (drinking and  
108 wastewater) and liquid medium swabs (eswabs).

### 109 ***Laboratory analyses***

#### 110 ***Microbiological cultivation***

111 Human and environmental samples were collected and stored at 4 °C during transport to  
112 the laboratories and processed within 48 hours.

113 All human samples (anal, vaginal and wound swabs) were streaked onto MacConkey agar  
114 plates (Oxoid Deutschland GmbH, Wesel, Germany) with a 10 µg imipenem disk  
115 (bioMérieux SA, Marcy-Étoile, France) and selective agar plates, e.g. CHROMagarESBL  
116 (Oxoid Deutschland GmbH, Wesel, Germany).

117 All Oxoid RODAC™ contact plates (∅ 55 mm, Becton Dickinson, Heidelberg, Germany)  
118 were used for environmental surface sampling and incubated at 36 ± 1 °C for 48 hours.

119 All environmental liquid medium swabs (eSwab; Copan Diagnostics, Italy) were streaked  
120 on Columbia agar plates containing 5 % sheep blood (Becton Dickinson), MacConkey  
121 agar plates (Oxoid Deutschland GmbH, # PO5002A), and selective agar plates, e.g.  
122 CHROMagar™ ESBL (bioMérieux SA, Marcy-Étoile, France; # 43481) and incubated at  
123 37 ± 1 °C for 48 hours. In addition, casein soy flour peptone broth (CASO-broth) (Merck  
124 KGaA, Darmstadt, Germany) was inoculated with the same swabs and incubated at 37 ±  
125 1 °C for 24 hours.

126 The disinfectant wipes, socks, and knitted hats of the newborns were put into 200 ml  
127 CASO-broth with tween saponin histidine cysteine (TSHC +3% Tween Merck, # 8.22187;  
128 +3 % saponin, Roth, # 5185.1; +0.1% histidine Merck, # 1.04351; +0.1 % cysteine, Merck,  
129 #. 1.02839) and homogenised for 60 seconds (Stomacher 400, Seward Limited, West  
130 Sussex, United Kingdom). First, 2 x 100 ml of the extract was filtered (membrane-filter  
131 Millipore Hydrosol, 250 ml cup) followed by the filtration of 100 ml 0.9 % NaCl to rinse the  
132 additives off the filter. The filter was then placed on CHROMagar™ ESBL and MacConkey  
133 agar plates and incubated at 37 °C for 24 – 48 hours. In addition, 100 µl of each  
134 concentration of a dilution series in 0.9 % NaCl was plated on CHROMagar™ ESBL and

135 MacConkey agar plates. The emulsions were treated in the same manner as the wipes,  
136 except that shredding was not necessary.

137 For the water samples, approximately 100 ml were collected in a sterile polystyrene cup  
138 (# 225170, Greiner, Frickenhausen, Germany) and filtered through a sterile nitrocellulose  
139 membrane filter (pore size  $0.45 \pm 0.02 \mu\text{m}$ ,  $\varnothing$  47 mm, black grid from Millipore, # EZHAWG  
140 474), according to Schulz and Hartung (18). After filtration, the membrane was placed on  
141 selective CHROMagar™ ESBL and MacConkey agar plates.

142 In addition, 100  $\mu\text{l}$  of each dilution of a dilution series in 0.9 % NaCl was plated on  
143 CHROMagar™ ESBL and MacConkey agar.

#### 144 *Identification and susceptibility testing of ARB*

145 All *Enterobacterales* detected on CHROMagar™ ESBL were identified via API 20 E V4.1  
146 apiweb™ (bioMérieux SA, Marcy-Étoile, France) and matrix-assisted laser  
147 desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS, bioMérieux SA,  
148 Marcy l'Étoile, France) using Myla® software, VITEK® MS-CHCA matrix (#411 071,  
149 bioMérieux) and disposable targets (# 410 893, bioMérieux).

150 Susceptibility to antibiotics was tested using the Micronaut-S MDR MRGN-Screening 3  
151 system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-  
152 Hersel, Germany) and results were interpreted using EUCAST criteria.

#### 153 *Testing of disinfectant tolerance*

154 To detect a possibly increased tolerance of the *K. oxytoca* isolates towards disinfectants,  
155 surface disinfectant and the disinfecting general-purpose detergent which had been used  
156 in the washing machines were both examined according to the regulations (7 and 9) of  
157 the Disinfectant Commission of the Verbund für Angewandte Hygiene e.V. (VAH) (19).  
158 The VAH method 7 was used to determine the minimal inhibitory concentration of the

159 environmental *K. oxytoca* strains in comparison to a Gram-negative reference strain  
160 *Pseudomonas aeruginosa* DSM 939 (20). The VAH method 9 respectively EN 13727 is a  
161 quantitative suspension test which was used to identify efficacy gaps, taking into account  
162 the concentration-time-relation, interfering substances and temperature of the  
163 disinfectants (21-23). The surface disinfectant used was based on quaternary ammonium  
164 compounds and alkylamine (formulation: didecyldimethylammoniumchlorid 12.5 g/100 g;  
165 bis-(aminopropyl)laurylamine 1.5 g/100 g). The laundry disinfectant was based on  
166 peracetic acid (formulation: 40 – 80 ppm peracetic acid; 2.5 – 5 g/l).

#### 167 Molecular resistance characterization

##### 168 *Molecular characterization of ESBL-genes*

169 Possible ESBL-production was confirmed by Polymerase Chain Reaction (PCR). Five  
170 specific primer sets were used to detect  $\beta$ -lactamase-encoding genes belonging to the  
171 *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub> families (24-27). The PCR products were visualised by gel  
172 electrophoresis on a 1 % agarose / TBE gel and stained with Midori Green (Biozym  
173 Scientific GmbH, Hessisch Oldendorf, Germany). The resulting amplicons were purified  
174 using an innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) according to  
175 the manufacturer's recommendations. Custom-sequencing was performed by Microsynth  
176 (Göttingen, Germany). The nucleotide sequences were analyzed using Chromas 2.6.5.

##### 177 *Plasmid characterization (Inc-typing)*

178 To distinguish the Inc-type of possible plasmids, two to three colonies of a fresh culture of  
179 all *K. oxytoca* isolates were resuspended in 100  $\mu$ l nuclease free water, heated to 95 °C  
180 for 10 min and centrifuged at 14,000 g for 5 min. The supernatant was carefully removed  
181 and used for PCR. The PCR mixture contained 2 x OneTaq Master mix (New England  
182 Biolabs) and 0.2  $\mu$ M of each primer (Table 1) (28-30).



183 The PCR conditions were set on 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s,  
184 60 °C for 30 s and 68 °C for 1 min. The final extension was performed at 68 °C for 5 min.  
185 The PCR products were analyzed using agarose gelelectrophoresis on a 1 %  
186 agarose/TBE gel, and stained with Midori Green (Biozym Scientific GmbH, Hessisch  
187 Oldendorf, Germany).

#### 188 *Plasmid conjugation testing*

189 The possibility of plasmid transfer via conjugation was analyzed. All *K. oxytoca* as well as  
190 wild type *E. coli* strains were streaked onto LB-agar containing a) 20 mg/l chloramphenicol  
191 and b) 20 mg/l chloramphenicol and 4 mg/l cefotaxime. *E. coli* strains that grew on LB-  
192 agar containing only chloramphenicol, but not on LB-agar containing both  
193 chloramphenicol and cefotaxime, were used for the following conjugation assay. For the  
194 pre-culture one colony of a fresh culture of all isolated *K. oxytoca* strains as donors and  
195 different wild type *E. coli* strains (recipients) were used for inoculation of 5 ml lysogeny  
196 broth (LB) and incubated at 37 °C overnight with shaking. Next, 5 ml fresh LB was  
197 inoculated with 100 µl of the donor (*K. oxytoca*) and 1 ml of the recipient (*E. coli*) and  
198 incubated at 37 °C for overnight with shaking. After incubation 1 ml of each culture was  
199 centrifuged at 14.000 rpm for 3 min. Afterwards the supernatant was discarded and the  
200 pellet was resuspended using 100 µl Milli Q. The whole suspensions, as well as 100 µl of  
201 the cultures were plated onto selective LB-agar containing 20 mg/l chloramphenicol and  
202 4 mg/l cefotaxime and incubated at 37 °C overnight. Grown colonies were streaked onto  
203 Columbia sheep blood agar and analyzed via MALDI TOF-MS.

#### 204 *Molecular typing*

205 All *K. oxytoca* isolated from clinical samples and environmental sources were typed by  
206 the German National Reference Centre (GNRC) using PFGE according to Tenover et al.

207 (31) and modified according to Ribot et al. (32). In addition, MLST of all *K. oxytoca* strains  
208 was conducted. Briefly, bacteria were cultivated on Columbia blood agar plates at 35 °C  
209 overnight. After resuspension of two to three colonies in 80 µl PCR water, the samples  
210 were heated to 95 °C for 5 min and centrifuged at 14,000 g for 5 min. The supernatant  
211 was used for PCR according to the protocol described by Herzog et al. (33). The PCR  
212 products were purified by extraction from a 1 % agarose gel using the GeneJET Gel  
213 Extraction Kit (Fisher Scientific GmbH, Schwerte, Germany) according to the  
214 manufacture's recommendations and sequenced by GATC Biotech AG (Konstanz,  
215 Germany). Assignment to the sequence type (ST) was performed via the specific MLST  
216 website (<https://pubmlst.org/koxytoca/>).

217

## 218 **Results**

### 219 ***Colonization with K. oxytoca in humans***

220 Within 14 months, 27 children were colonized (but not infected) with *K. oxytoca* according  
221 to CDC definitions (34). The occurrence of *K. oxytoca* varied from sensitive isolates with  
222 no ESBL -activity to ESBL-producing *K. oxytoca* and the particular ESBL-producing *K.*  
223 *oxytoca* PFGE type-00531. Fourteen children were tested positive for the ESBL-*K.*  
224 *oxytoca* PFGE type-00531.

225 *K. oxytoca* was mostly identified in rectal swab screening samples. In 9 out of 24 newborns  
226 *K. oxytoca* was also detected in throat swap samples. As an exception, a 4-year-old boy  
227 in the ICU, who never had any direct contact with the PNC, also showed rectal colonization  
228 with PFGE type-00531/ST201 ESBL-*K. oxytoca* in March 2013.

229 All ESBL-producing *K. oxytoca* strains were resistant to piperacillin/tazobactam, with a  
230 minimum inhibitory concentration (MIC) up to > 64/4 mg/l, to cefotaxime (MIC ≥ 64 mg/l),

231 ceftazidime (MIC > 128 mg/l) and ciprofloxacin (ranging from MIC 2 to >2 mg/l) and can  
232 be classified as multidrug-resistant according to Magiorakos et al. (35). Figure 1  
233 summarises the occurrence of *K. oxytoca* isolates over time in the PNC, ICU, and on four  
234 different wards.

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

239 During an extended screening of mothers and healthcare workers, a total of 695 swabs  
240 (vaginal and rectal) from 428 persons were obtained on the Obstetrics Ward. The  
241 screening identified four mothers colonized with *K. oxytoca*, five mothers colonized with  
242 ESBL-producing *E. coli*, and one with ESBL-producing *K. pneumoniae*. None was positive  
243 for the special PFGE type-00531 ESBL-producing *K. oxytoca*. A transmission between  
244 mother and newborn was not documented.

245

#### 246 ***Environmental monitoring***

247 All environmental samples taken during the first and second on-site inspections were  
248 tested negative for *Enterobacterales* and non-fermenting organisms. During the second  
249 on-site sampling in October 2012, only low concentrations of Gram-positive skin and  
250 environmental bacteria were present. However, during the third on-site inspection in June  
251 2013, sampling was conducted using native samples (drinking and wastewater) and liquid  
252 medium swabs (eswabs). Thus, *Enterobacterales* as well as non-fermenting bacteria were  
253 detected. All *K. oxytoca* isolates were identical ESBL-producing strains and belonged to

254 PFGE type-00531 (Table 2). In the following these *K. oxytoca* strains are termed  
255 “environmental”.

256 Water-associated bacteria such as *P. aeruginosa*, *Serratia* spp., *Enterobacter* spp., *K.*  
257 *pneumoniae*, and *S. maltophilia* were detected in the siphons of hand wash basins.  
258 Identical clones of *K. oxytoca* PFGE type-00531/ST201 were isolated from the siphons of  
259 two sinks in the healthcare workers’ staff room and in the room used for cleaning and  
260 disinfection. The same clone was also isolated in high concentrations from samples of  
261 residual water in the rubber seal and one swab sample (in addition to *P. aeruginosa*) from  
262 the detergent compartment of one of the two washing machines (Table 2), located in the  
263 ground floor of the same ward.

264 Following identification of the washing machine as potential reservoir for this specific *K.*  
265 *oxytoca* clone, newborn clothing (hats and socks) that had been washed in this machine  
266 were microbiologically analysed. *K. oxytoca* PFGE type-00531/ST201 of the same  
267 specific clone was isolated with a total count of  $> 10^9$  CFU / ml.

268 All ESBL-producing *K. oxytoca* PFGE type-00531/ST201 found in the residual water and  
269 environmental samples were also resistant to piperacillin/tazobactam, with a minimum  
270 inhibitory concentration (MIC) ranging from 8/4 mg/l up to  $> 64/4$  mg/l, to cefotaxime (MIC  
271  $> 2$  mg/l), ceftazidime (64 mg/l) and ciprofloxacin (MIC ranging from 2 mg/l to  $> 2$  mg/l)  
272 (Table 3).

273 The presence of the ESBL gene CTX-M-15 was confirmed by sequencing in all human  
274 and environmental strains. The transfer of putative plasmids that harboured these genes  
275 under laboratory conditions into wild type *E. coli* strains via conjugation was not  
276 successful. In addition, no amplificates for *inc* (*A/C*, *L*, *HI2*, *M*, *I1- $\alpha$* , *N*, *FIIk*, *FIA*, *FIB*, *FII*)

277 genes were obtained from the strains indicating that the CTX-M genes might be  
278 chromosomally located as described by Rodríguez et al. (36).

279

### 280 ***Epidemiological links***

281 Retrospective analysis demonstrates that only newborns who had worn clothing that had  
282 been washed in the in-house washing machine were colonised with the redundant *K.*  
283 *oxytoca* clone. Although the siphons of the staff sinks were also identified as a possible  
284 reservoir, no staff members were identified as carriers or spreaders of ESBL-  
285 *Enterobacterales* at the time of screening.

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

289

### 290 ***Evaluation of a possibly increased disinfectant tolerance of the K. oxytoca strains***

291 The environmental *K. oxytoca* isolates showed no increased tolerance to the surface  
292 disinfectant based on quaternary ammonium compounds and alkylamine. The minimal  
293 inhibitory alkylamine concentration on the *K. oxytoca* strains was identified as max.  
294 0.003% and 0.0075% on the reference strain *Pseudomonas aeruginosa*. Furthermore, the  
295 examination according to VAH method 9 showed a  $\geq 5 \log_{10}$  CFU reduction of the *K.*  
296 *oxytoca* strains at a concentration of 0.5% and 15 min in the presence of interfering  
297 substances (dirty conditions – 3,0 g/l bovine serum albumin + 3,0 ml/l sheep erythrocytes).

298 The VAH-recommended concentration and application time is 0.5% over 60 minutes (37).

299 *K. oxytoca* isolates from the environment showed no increased tolerance to the laundry  
300 disinfectant based on peracetic acid. The minimal inhibitory concentration of the *K.*

301 *oxytoca* strains and the *P. aeruginosa* reference strain was identified as 5 g/l. The analysis  
302 according to VAH method 9 (in the presence of 2.75g/l laundry disinfectant with an  
303 exposure time of 15 min under dirty conditions at 20°, 40° and 60°C) demonstrated that  
304 the *K. oxytoca* strains were more susceptible than the Gram-negative reference strain *P.*  
305 *aeruginosa* DSM 939. At a temperature of 60 °C all *K. oxytoca* strains showed a  $\geq 5 \log_{10}$   
306 reduction.

307

### 308 ***Hygienic containment measures and control management strategies***

309 A task force was reinforced to manage the control interventions for prevention of the  
310 colonization of further patients and to identify possible sources of the suspected *K.*  
311 *oxytoca* strain.

312 The management procedures can be divided into three main phases until the  
313 colonisations were under control and the source could finally be identified. Therefore,  
314 during each phase step-by-step approaches with different strategies were necessary in  
315 parallel to sampling during the whole report period (Figure 1, Table 4).

316

### 317 ***Long-term clinical effects/concomitant infection control interventions***

318 After the washing machine had been taken out of use, no further colonization of newborns  
319 with *K. oxytoca* has been detected to this day. All garments worn by the newborns and  
320 children were laundered by a professional external hospital laundry service after the  
321 outbreak. The two colonized sinks were replaced by sinks with specialised thermosiphon  
322 systems. As a further consequence of the prolonged cluster process, the existing infection  
323 control measures (isolating colonized patients, enforcing hand hygiene measures, and  
324 cleaning the ward, particularly the sinks and equipment) were reinforced via extra training.

325 The screening plan for all newborns and children, and all measures implemented within  
326 the containment program, continues to be maintained.

327

## 328 **Discussion**

### 329 ***Reservoirs and transmission routes of waterborne pathogens in healthcare*** 330 ***systems***

331 When the first five cases of colonization with *K. oxytoca* had been detected between  
332 August and October 2012, it was suggested to be a person-to-person transmission.  
333 Although this has not been reported for *K. oxytoca*, Price et al. (38) showed that, in the  
334 presence of standard infection control measures, healthcare workers (HCWs) were  
335 frequent sources of *Staphylococcus aureus* transmission. Furthermore, Heudorf et al. (39)  
336 mentioned that pathogens classified by the WHO as highest priority can be found on staff  
337 gowns, which can be the starting point of transmissions (40).

338 In this case the occurrence of *K. oxytoca* isolates continued over a 1-year period which  
339 indicated a reservoir in the environment rather than hospital personnel or mothers. Despite  
340 the strict implementation of control management and containment measures, newborns  
341 and children continued to acquire the cluster organism (PFGE type-00531/ST201) which  
342 had never been detected before by the GNRC. The single database strain was isolated in  
343 the Netherlands in 2018 (41).

344

### 345 ***Washing machine***

346 In this case, the particular *K. oxytoca* strain was not only detected in two sinks but also in  
347 a domestic washing machine with integrated dryer. Even recently published articles  
348 reviewing the main water-associated reservoirs in hospitals do not consider the washing

349 machine as a potential hazard in a clinical environment (4, 5). However, in our report the  
350 knitted clothes of patients were washed in an in-house washing machine designed for  
351 household use and enabling a washing temperature of 65°C. It has been shown, that  
352 resistance genes as well as different microorganisms can persist in domestic washing  
353 machines (17, 42).

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

364 It remains unclear how the washing machine was contaminated. However,  
365 *Enterobacterales* can survive in wet environments for an extended period of time (1),  
366 especially waterborne bacteria such as *P. aeruginosa* and *Klebsiella* strains, which have  
367 the ability to survive in a viable but not culturable state. Similarly, their environmental  
368 stability is supported by the formation of biofilms to enhance their chances of multiplication  
369 and horizontal gene transfer (43). Zhang et al. (44) discusses that biofilm detachment is  
370 promoted by disinfectants and thus affects the overall bacterial antibiotic resistance of  
371 microbes in tap water.



372 Furthermore, Rehberg et al. (17) demonstrated that ARB can survive the washing  
373 process. In tests with *P. aeruginosa* outbreak strains, even the use of temperatures above  
374 50 °C could not achieve a secure reduction of those strains. Even though a 65 °C washing  
375 program had been used for every wash cycle, it is likely that the temperature in the area  
376 of the rubber mantle or the rubber door seal would have been much lower, providing an  
377 optimal humid environment and nutrient supply for the growth of Gram-negative  
378 microorganisms (43, 45). Moreover, the occasional use of washing machines at low  
379 temperatures supports the formation of biofilms (14).

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

386

### 387 **Conclusion**

388 While previous studies have implicated sinks as potential reservoirs for clusters of  
389 infection caused by *K. oxytoca*, this report focuses on the washing machine. The extensive  
390 time period of the outbreak shows that while facing several potential environmental  
391 reservoirs, a multi-dimensional containment approach is necessary. Here, the approach  
392 included reinforcement of infection control policies such as hand hygiene, contact  
393 precautions, isolation, admission/routine rectal screening and clear delineation between  
394 handwashing sinks and sinks used for other purposes. However, only after the washing  
395 machine was removed and only disposable caps and socks were used the ongoing

396 colonization was terminated. The results suggest that, for prompt management of  
397 outbreaks or colonization clusters, the choice of environmental sampling points and the  
398 use of an effective method should take the ecological properties of the causative strain  
399 into account. Indeed the environmental strain had not been found before the third-on-site  
400 inspection, as the use of e-swabs allowed a better detection.

401 In addition, the study implies that changes in washing machine design and processing are  
402 required to prevent accumulation of residual water where microbial growth can occur and  
403 contaminate clothes. Furthermore, the use of professional washing machines and the  
404 routine check with a temperature logger is an urgent requirement.

405 In summary, the present study shows that, in situations where an increase in colonization  
406 is observed, non-professional washing machines, if used, and clothes should be assessed  
407 and investigated as potential reservoirs and vectors for transmission.

408

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413

#### 414 **Conflicts of interest**

415 None declared.

416

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- 564



565 **TABLE CAPTIONS**

566

567 **TABLE 1** Primers used for Inc-typing.

568

569 **TABLE 2** Occurrence of Gram-negative *Enterobacterales*, non-fermenting organisms,  
570 and ESBL-producing *K. oxytoca* PFGE type-00531 isolates in environmental samples  
571 obtained during on-site inspection of risk areas.

572

573 **TABLE 3** Occurrence of Gram-negative *Enterobacterales*, non-fermenting organisms,  
574 and ESBL-producing *K. oxytoca* PFGE type-00531 isolates in environmental samples  
575 obtained during on-site inspection of risk areas.

576

577 **TABLE 4** Hygienic measures and control management procedures.

578 **TABLES**579 **TABLE 1** Primers used for Inc-typing.

IncHI2_fw	TTT CTC CTG AGT CAC CTG TTA ACA C	Carattoli et al. (29)
IncHI2_rev	GGC TCA CTA CCG TTG TCA TCC T	Carattoli et al. (29)
IncA/C_fw	GAG AAC CAA AGA CAA AGA CCT GGA	Carattoli et al. (29)
IncA/C_rev	ACG ACA AAC CTG AAT TGC CTC CTT	Carattoli et al. (29)
IncL_fw	CGG AAC CGA CAT GTG CCT ACT	Carattoli et al. (30)
IncM_fw	GGA TGA AAA CTA TCA GCA TCT GAA G	Carattoli et al. (30)
IncL/M_rev	GAA CTC CGG CGA AAG ACC TTC	Carattoli et al. (30)
IncI1- $\alpha$ _fw	CGA AAG CCG GAC GGC AGA A	Carattoli et al. (29)
IncI1- $\alpha$ _rev	TCG TCG TTC CGC CAA GTT CGT	Carattoli et al. (29)
IncN_fw	GTC TAA CGA GCT TAC CGA AG	Carattoli et al. (29)
IncN_rev	GTT TCA ACT CTG CCA AGT TC	Carattoli et al. (29)
IncFIk_fw	TCT TCT TCA ATC TTG GCG GA	Villa et al. (28)
IncFIk_rev	GCT TAT GTT GCA CRG AAG GA	Villa et al. (28)
IncFIA_fw	CCA TGC TGG TTC TAG AGA AGG TG	Carattoli et al. (29)
IncFIA_rev	GTA TAT CCT TAC TGG CTT CCG CAG	Carattoli et al. (29)
IncFIB_fw	TCT GTT TAT TCT TTT ACT GTC CAC	Villa et al. (28)
IncFIB_rev	CTC CCG TCG CTT CAG GGC ATT	Villa et al. (28)
IncFIi_fw	CTG ATC GTT TAA GGA ATT TT	Villa et al. (28)
IncFIi_rev	CAC ACC ATC CTG CAC TTA	Villa et al. (28)

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582 **TABLE 2** Occurrence of Gram-negative *Enterobacterales*, non-fermenting organisms,  
 583 and ESBL-producing *K. oxytoca* PFGE type-00531 isolates in environmental samples  
 584 obtained during on-site inspection of risk areas.

Room	Sampling point	CFU / ml or total Gram-negative bacteria count	Microbiological differentiation	CTX-M	K. oxytoca PFGE type-00531 / ST201
Nursing care room, storage rooms	Working basin, siphon	2,1 x 10 <sup>5</sup>	<i>S. marcescens</i>	-	
	Hand wash basin, siphon water	1.6 x 10 <sup>6</sup>	<i>K. oxytoca</i> <i>R. terrigena</i> <i>E. cloacae</i> complex	positive negative negative	yes
	Hand wash basin drainage hole cover	scattered/swab (no CFU/ml)	<i>E. cloacae</i> complex <i>K. pneumoniae</i>	negative negative	
Staff toilet	Hand wash basin, fresh water	not evaluable/100 ml	Gram-negative non-fermenting rods	-	
	Hand wash basin, siphon water	1.5 x 10 <sup>4</sup>	<i>P. aeruginosa</i> <i>S. maltophilia</i>	-	
	Toilet, water	1.0	<i>S. maltophilia</i>	-	
Staff room	Water from the kitchen sink siphon	3.0 x 10 <sup>5</sup>	<i>K. oxytoca</i> <i>P. aeruginosa</i>	positive negative	yes
	Water reservoir tank, espresso machine	10.0 CFU/50 ml	<i>S. maltophilia</i>	-	
Hygiene sluice, PNC	Hand wash basin, siphon water	1.3 x 10 <sup>4</sup>	<i>P. aeruginosa</i> , Gram-negative non-fermenting rods	-	
Basement, laundry room	Tumble dryer, water tray	not evaluable/50 ml	Gram-negative non-fermenting rods	-	
	Washing machine 1, residual water, rubber mantle	>10 <sup>6</sup> ESBL 3.9 x 10 <sup>2</sup>	<i>K. oxytoca</i>	positive	yes
	Washing machine 1, detergent compartment	not evaluable/swab	<i>K. oxytoca</i> <i>P. aeruginosa</i>	positive negative	yes
	Washing machine 2, detergent compartment	not evaluable/swab	<i>P. aeruginosa</i>	-	
Laundry	Hat 1	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes
	Hat 2	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes

	Hat 3	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes
	Hat 4	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes
	Socks 1	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes
	Socks 2	> 10 <sup>9</sup>	<i>K. oxytoca</i>	positive	yes

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587

588 **TABLE 3** Occurrence of Gram-negative *Enterobacterales*, non-fermenting organisms,  
589 and ESBL-producing *K. oxytoca* PFGE type-00531 isolates in environmental samples  
590 obtained during on-site inspection of risk areas.

no	ID	Sample Type	Pipera- cillin (MIC)	Piperacillin/ Tazobactam (MIC)	Cefo- taxim (MIC)	Cef- tazidim (MIC)	Imi- penem (MIC)	Mero- penem (MIC)	Ami- kacin (MIC)	Cipro- floxacin (MIC)
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 washbasin 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 washbasin 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 washbasin 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)

591

592

593 **TABLE 4** Hygienic measures and control management procedures.

Hygienic measures and control management strategies	
1) 04 - 08 / 2012	<ul style="list-style-type: none"> <li>- Environmental monitoring including sampling of siphons, sinks, and showers via contact plates</li> <li>- Continuation of admission screening and weekly routine screening, implementation of a second newborn-screening 48 hours post-partum</li> <li>- Screening of all mothers on admission and on discharge</li> <li>- Monitoring of pathways of newborns from birth to admission in the perinatal centre</li> <li>- Maintenance of incubator isolation until two-time negative screening results</li> <li>- Advanced training of health care workers with special focus on toilet and hand hygiene</li> <li>- Microbiological analyses of vaginal ultrasound probes and heated towels used for the primary care of the newborns</li> <li>- Renovation/decontamination of ward rooms, removal of unused sinks</li> <li>- Installation of new wall-mounted disinfection dispensers</li> <li>- Clinical procedure screening for any cross-contamination possibilities</li> <li>- Feeding of newborns with only precooked single nurture packages</li> <li>- Use of sterile water for bathing of newborns</li> </ul>
2) 08 - 10/2012	<ul style="list-style-type: none"> <li>- Screening of all health care workers working in the perinatal centre, intensive care unit and the obstetrics department</li> </ul>
3) 10/2012 - 06/2013	<ul style="list-style-type: none"> <li>- Extended environmental sampling from different areas on the ward and functionally linked areas in the hospital. Identification and sampling of specific risk factors (e.g. washing machine)</li> <li>- Training of staff in the correct handling of the disinfectant wipes</li> <li>- Training of health care workers in correct disinfection and cleaning of incubators</li> <li>- Preparation of disinfectant wipes by a newly validated/approved preparation machine</li> <li>- Construction measures to provide work space for the hygienic processing of incubators and ensure sufficient storage capacities</li> <li>- Marking of all thermometers (rectal and commercially available thermometers) as well as incubators for traceability</li> <li>- Intensive audit of all procedures running on ward during routine operations</li> </ul>

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596 **FIGURE CAPTIONS**

597

598 **FIG 1** Course of the outbreak with different *K. oxytoca* strains within one year and their  
599 distribution on different wards (PNC: perinatal centre, ICU: intensive care unit).

