

Microbial competition in environmental nosocomial reservoirs and diffusion capacity of OXA48-*Klebsiella pneumoniae*: potential impact on patients and possible control methods

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Keywords

Control; hospital-reservoir • OXA48-*K. pneumoniae* • VIM-*Enterobacteriaceae*

Summary

Background. We have found clusters of *Klebsiella pneumoniae* with OXA48-carbapenemase cases in some hospital rooms, and decided to investigate whether bathroom siphons could be a reservoir for OXA48 bacteria, as occurs with *K. oxytoca* with other types of carbapenemases.

Methods. We evaluated the microbial competition between strains with OXA48 and VIM carbapenemases, in diluted nutrient-broth, on a slime germ-carrier. We compared the number of colonies at 5 and 10 days on the contaminated carriers with one or two strains. We evaluated the dissemination of *K. pneumoniae* with carbapenemase OXA48 or VIM from thumbs and index fingers of volunteers, to standard surfaces (20 glass germ-carrier by each volunteer). After, we counted the number of microorganisms on each carrier. Microbiological weekly studies of faecal microbiota of all patients were obtained in Traumatology and Oncology. Moreover, we studied samples of the sink in their rooms. PCR and MLST sequence-type was determined in all *K. pneumoniae* diagnosed from patients and sinks.

Results. A large possibility of diffusion from contaminated hands, which continue to transmit high numbers of microorganisms after more than 10 successive surface contacts, was highlighted; OXA bacteria were more persistent than VIM bacteria. Microbial competition studies showed that VIM bacteria are inhibited by OXA ones. These observations can explain the concentration of cases of *K. pneumoniae* OXA48 in some rooms in Traumatology and Oncology, producing a significant OR between rooms with OXA48-bacteria-contaminated siphons and other rooms (3.1 and 3.3 respectively). Risk was lowered after changing or disinfecting (heat plus chlorinated disinfectant) the contaminated siphons. Siphon colonization by VIM bacteria was not related with human infections by similar microorganisms.

Conclusions. Bathroom siphons can be a reservoir for *K. pneumoniae* OXA48 and lead to outbreaks. Outbreaks can be controlled by replacement or heat plus chemical treatment of the sink-siphons.

Introduction

Environmental contamination in hospitals has become increasingly important given our patients' higher susceptibility to infection because of several concurrent causes such as increasing age, multiple pathologies, longer hospital stays, more and longer instrumentation periods or treatments with broad spectrum antibiotics. These circumstances allow colonization, and, in some cases, also infection by multi-drug resistant microorganisms (MDRM) [1], especially if the latter have good/strong environmental resistance in some hospital reservoir [2-4].

Many publications have reported an increased risk for colonization or infection in patients admitted to rooms where the previous patient was colonized (or infected) by an MDRM, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus*, *Enterobacteriaceae* with extensive spectra of beta-lactamase (ESBL) or carbapenemases [5-10]. This

is mainly due to a failure in inter-patient cleaning or disinfection of these rooms that results in the non-elimination of some surface microorganisms that can then colonize or infect the next patient to use the surface [11-16]. Usually cleaning or disinfection is successful and our surface studies consistently indicate no MDRM in the rooms that had held colonized patients. Therefore, if a new patient is contaminated, it must be due to microorganisms being carried by healthcare workers who have not followed contact precautions or from microorganism reservoirs that are difficult clean during the inter-patient room cleaning/disinfection [17, 18].

Reservoirs have sometimes been seen with hydrophilic bacteria like *P. aeruginosa* or *B. cepaciae* or *Enterobacteriaceae* as KPC-*K. pneumoniae* [19] (but not among OXA-*K. pneumoniae*) or VIM-*Klebsiella oxytoca* carbapenemase (a metallo-β-lactamase that gives resistance to most antibiotics used in our hospitals). These *Klebsiella* have been detected in outbreaks in different tertiary hospitals and are associated with sink contamination; hence,

according to Lowe 2012 [2]: “Sinks should be considered as potential reservoirs when clusters of infection caused by *K. oxytoca* are investigated.” However, the pathogenic mechanism explaining the transmission from this reservoir to the patient remains unclear.

A similar reservoir has not been reported for *K. pneumoniae* with carbapenemase type OXA48. Siphons in sinks can harbor biofilms, which, in addition to hampering disinfection, can facilitate survival by microorganisms. Therefore, this added factor should be considered when assessing antimicrobial actions to achieve surface bacterial removal [20].

Since we found clusters of these cases in some rooms in our hospital, we decided to investigate whether the sinks in the patient’s rooms were acting as reservoirs.

Material and methods

A) LABORATORY: IN VITRO STUDIES

Materials:

- a. Microorganisms: collected from clinical samples or weekly rectal swabs of patients from La Paz University Hospital (ICUs and Services with at least one case of OXA48-*K. pneumoniae*).
 - I) Two strains of *K. pneumoniae* with OXA48-carbapenemase (strains with international dissemination such as sequence-types ST-11 and ST-405, which are involved in more than 75% of colonization cases in our hospital).
 - II) Six strains of microorganisms with VIM-carbapenemase (one *K. pneumoniae*, two *K. oxytoca*, two *Enterobacter cloacae* and one *Serratia marcescens*).
- b. A surface-germ-carrier (a standard-sized, easy-to-manipulate surface model [21]): rectangular glass cover-slides sized 12 x 15 mm. The number of bacteria in 10 µl of nutrient broth (after 24 h incubation at 37°C) or on the germ-carrier contaminated with 10 µl of this broth was very similar (6.65 log₁₀ vs 6.48-6.7), indicating excellent recovery by the inoculums on these germ-carriers.
- c. A slime-germ-carrier (to favor slime formation): A 5-cm long brush for test tubes that has a metal center with circumferential bristles at different heights.

Methods:

1. Study of microbial competition between strains with OXA48 and (or) VIM carbapenemases on a slime germ-carrier.
 - Brush, with a large surface in the form of close-sitting bristles to promote the formation of slime were introduced in a diluted nutrient broth (Nutrient broth, Difco, diluted 10 times in sterile distilled water). Six tubes, each with a brush and 10 ml of this nutrient broth were prepared. In the 1st and 4th tubes one colony of OXA48-*K. pneumoniae* was introduced; in the 2nd and 5th, one colony of *S. marcescens* with VIM carbapenemase; and in the 3rd and 6th, a colony made up of both

species. On the 5th day, the transfer of carrier to a new nutrient broth for the first set was interrupted, when three carriers with 5-day old colonies adhered to them, were transferred to a nutrient broth diluted 10 times and centrifuged at 2,000 rpm for two min.

- Three 0.1 ml aliquots of the supernatant from each fifth-day carriers were extracted and plated them either directly on 4 McConkey plates, or after 1/100 or 1/10,000 dilutions to facilitate counts of colony forming colonies units (FCU). In parallel, other aliquots from these tubes were seeded (directly or diluted as above) on OXA-plates. After incubating the seeded plates at 37°C for 48 h, the FCU of the brush samples were counted. The difference between the number of FCU on McConkey and OXA-plates was estimated as the FCU-VIM recount. The second set of three tubes with individually, or jointly, seeded bacteria were transferred to new broth diluted in sterile water each day until the 10th day and then handled them the same as the first set on the 5th day, before counting surviving FCU.
2. Study of the adhesion and dissemination ability of these *K. pneumoniae* from hands to surfaces.

We cultured OXA48-*K. pneumoniae* (ST11) for 24 h in a nutrient broth at 37°C and 10 µl of the culture was removed to contaminate finger pads of five volunteers. The pad of the thumb was rubbed over the index finger, to achieve a uniform distribution of inoculum on both fingers. After allowing the fingers to air-dry for 15 min, one surface-germ-carrier was extracted, with flame-sterilized tweezers, and placed between the two contaminated fingers. Next, the volunteers pressed their fingers for one second to contaminate the first germ carrier and then deposited it into a test tube with 5 ml of nutrient broth with 0.5 g of sterile glass beads. Immediately, each volunteer took another surface-germ carrier between the same fingers and after contaminating it for one second, deposited it in another test tube (as was done with the first germ carrier). This operation was repeated with 18 additional surface-germ-carriers. After, all volunteers applied an alcohol-solution of proven efficacy on these bacteria to their hands. In all, 20 germ-carriers were contaminated by each volunteer, and then the test tubes with the germ carriers were centrifuged at 20,000 rpm for two min. We extracted three 0.1-ml aliquots of each supernatant and plated them directly (as well as after dilutions of 1/100 to 1/10,000, for improved microbial counts) on sterile McConkey plates. These were incubated for 48 h at 37°C and then the number of FCU in each was counted.

The above was repeated using VIM-*K. pneumoniae* instead OXA48-*Klebsiella*.

B) CLINICAL: EPIDEMIOLOGICAL AND MICROBIOLOGICAL STUDIES OF PATIENTS AND THEIR ROOMS

Weekly studies on the colonization of the fecal microbiota (swabs) of all patients admitted to two areas of

our hospital (Traumatology and Oncology) were performed in the period of study (18 and 12 months, respectively), because there had been cases of infection with OXA48-*K. pneumoniae* in some of these rooms. Moreover, we took samples from the sinks in each room with a sterile swab, either directly, if the siphon could be reached without removing the drain, or after disassembling it. We sent the samples to the Microbiology Service and they were processed as if they had some from the patients. If OXA48-*K. pneumoniae* was isolated, PCR determined the strain to which it belonged. The genetic relationships between the isolates of OXA48-*K. pneumoniae* were determined by automated repetitive-sequence-based PCR using the DiversiLab® system (bioMérieux). The multilocus sequence typing (MLST) – “sequence type” – was determined according to the Institute Pasteur scheme (<http://bigdb.web.pasteur.fr/klebsiella/klebsiella.html>).

This helped to assess whether or not there was a relationship between the strain isolated from the siphon and those from patients admitted to the room.

All the sink siphons were changed (in the same month) in the rooms of the oncological patients. However, this was not done in the rooms of the Traumatology, because it would have required construction work that could not have been done without closing the room to new admissions. These rooms were subjected to a chemical treatment with a surface-disinfectant comprised of a chlorinated product (4,000 ppm chlorine) and anionic surfactants. This product is currently used as a surface disinfectant in our hospital. We chose it because it has an oxidant with a surfactant that can be useful in eliminat-

ing both the microorganisms in the sinks and the slime that protects them. After we had treated the sinks by pouring 5 l of this product down each drain, we collected samples from the siphon to check whether the treatment had been successful. In cases where it had not, we increased the volume of the disinfectant poured down the sink by 5 l. In one room we failed to kill bacteria with this method, so we applied heat by using steam through a steam cleaner (Lavorwash®, model Starsteam, type HP58DS-M), which has been reported to destroy slime. Subsequently, we poured another 5 l of the same disinfectant down the sink. Thereafter, we took new microbiological samples, as in previous cases, to assess the efficacy of this combined heat and chemical disinfection method.

In the following 9 months we sent weekly samples from the patients of this hospital floor to the Microbiology Service and they were processed to investigate if they had some carbapemase microorganism. If OXA48-*K. pneumoniae* was isolated, PCR determined the strain to which it belonged. Finally, we took new samples from the sink in rooms where *K. pneumoniae* with OXA48 had been isolated.

Results

Microbial competition: in a media with scarce nutrient, and surfaces where slime can develop (Fig. 1), individual strains grew well, with similar increases in the number of FCU between days 5 and 10 (approximately $6 \log_{10}$). However, in the mixed cultures (*Serratia* plus *Klebsiella*) the OXA48-*K. pneumoniae* multiplied as

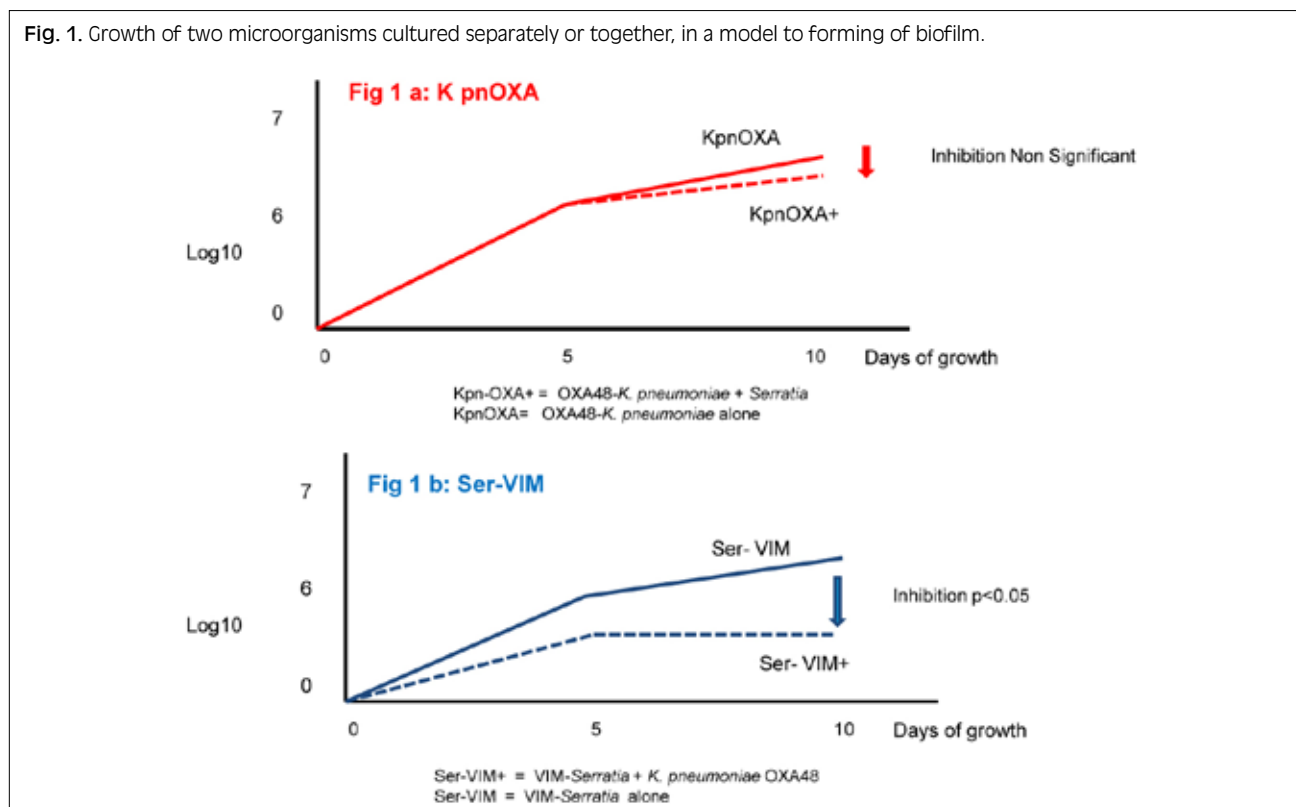
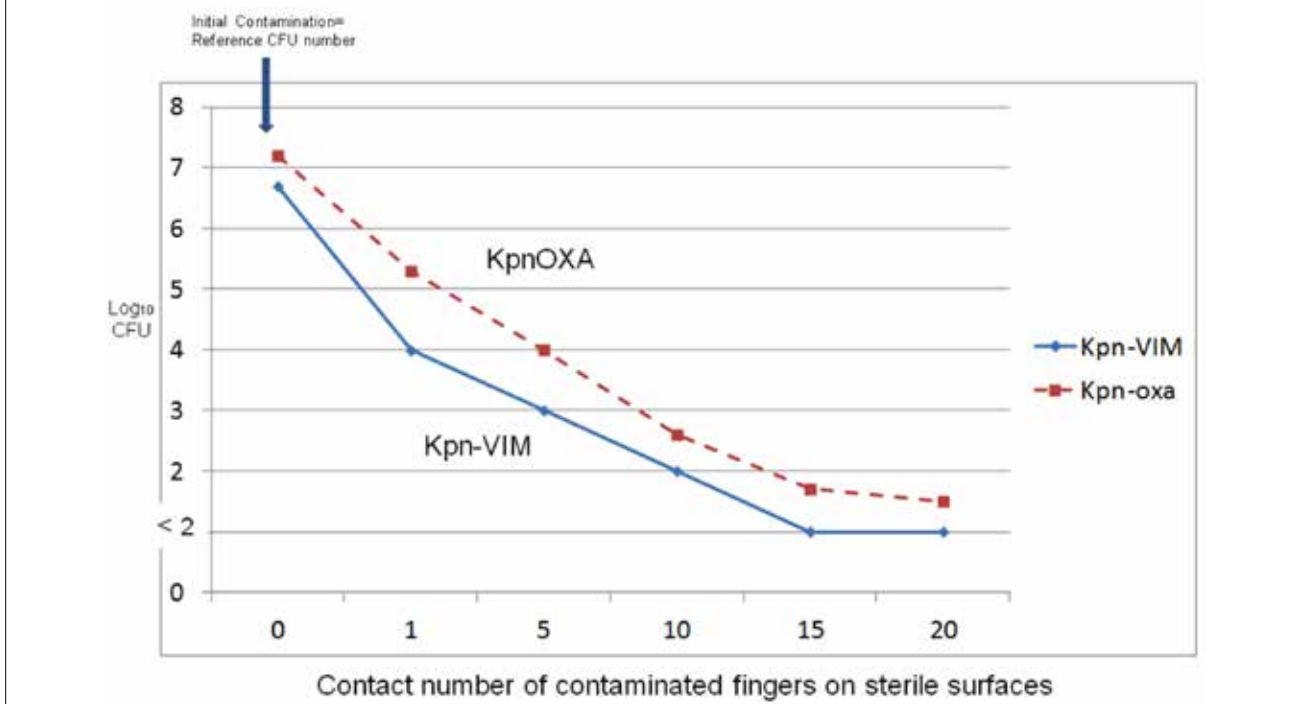


Fig. 2. Possibilities of dissemination of OXA or VIM *K. pneumoniae*, from human fingers (mean of CFU \log_{10} -reduction, after successive surface contacts).



if sown alone ($p > 0.05 = \text{NS}$), whereas VIM-*Serratia* growth slowed by one decimal logarithm, and the trend was a relative decline in percentage ($p < 0.05$).

Diffusion power: Figure 2 shows the large diffusion capacity of hand contamination, detecting a transmission of a high number of FCU after more than 20 successive contacts. So, a single subject could contaminate several patients if the contact was made without antiseptics. There was a difference between the OXA48 and VIM: the slope of the line describing the transfer from hands to surfaces is lower for VIM-*K. pneumoniae* than for OXA48, implying a greater diffusion capacity from hands contaminated with OXA48 than VIM-*K. pneumoniae* (higher number of contacts with more than 3 \log_{10} of microorganism survival: VIM, 5 contacts and OXA 8 contacts).

The Traumatology hospitalization area was studied in two steps (Tab. I): first from July (2013) to March (2014). There were 29 patients with OXA48 microorganisms, from these, 28 were OXA48-*K. pneumoniae*. All these bacteria were genotyped, except two. Moreover, OXA48 was only detected in sinks of 4 of the 21 rooms: room 1 (only in the last month, the other months it showed *Raoultella* with VIM), and room numbers 12, 18 and 20. In other rooms VIM microorganisms, such as *Citrobacter K. oxytoca*, *Enterobacter*, etc. or B-lactamase type ESBL bacteria, or microorganisms without antibiotic resistance, were detected.

If we accept contamination from the water reservoir as possible only if species and strain match in each room, this occurred in 10 of the 26 cases with OXA48-*K. pneumoniae*. However, none of the siphons colonized by

VIM matched the bacteria found in the patients in the same room (being different species it was not necessary to type them, see Table I). The number of studied patients was 463. The incidence of OXA48-*K. pneumoniae* in this period was 13.2% in the case of rooms with siphons positive for this bacterium and 4.7% if the siphons were negative. The OR (room with OXA48 to room without OXA48) was 3.1 (1.4-7.1; $p < 0.01$), that is, we have a suggestive coincidence (causality) between a water reservoir for specific rooms and their patients, but that is only true in the case of bacteria with OXA48, but not with VIM.

Drains were not changed in the Traumatology area because it would have required construction work. We therefore tried to treat contamination and slime with chlorine disinfectant (already used on surfaces). Pouring 5 l in the sinks of the 4 rooms, achieved success in the 12th room (where only *Serratia* with VIM had been detected) and the 20th (where *K. pneumoniae* with ESBL had been found), but rooms 1 and 18 continued to show OXA48. The application was increased to 10 l and success was obtained in room 18, but not in room 1. After treating the room 1 drain with steam under pressure, we poured 5 l of the same disinfectant and only found *Raoultella* with VIM (the bacteria that colonized this sink in the first months of the study, before it was colonized by OXA48).

The second step was following inpatients admitted to this floor during another 9 months (Fig. 3). The number of patients was 426. Cases were regularly distributed among the rooms, but strain ST11 was not found, and, practically, all cases were ST405. We compared

Tab. I. Summary of epidemiologic surveillance of bacteria with carbepenemases in Traumatology during 9 months (weekly cultures of patients).

	OXA microorganisms	VIM microorganisms
Number of patients with colonization	29	19
Species and strain isolated in patients	28 <i>K. pneumoniae</i> : ST405: 20 ST11: 5 strain 9: 2 strain 6: 1 ND strain: 2 1 <i>E. coli</i>	6 <i>K. pneumoniae</i> 1 <i>S. marcescens</i> 6 <i>K. oxytoca</i> 3 <i>E. coli</i> 3 <i>E. cloacae</i>
Number of sink-room with these bacteria	4	12
Species and strain isolated in these sinks	3 <i>K. pneumoniae</i> ST405 1 <i>K. pneumoniae</i> strain 9	1 <i>K. pneumoniae</i> 4 <i>S. marcescens</i> 3 <i>K. oxytoca</i> 3 <i>C. freundii</i> 3 <i>E. cloacae</i> 1 <i>R. planticola</i>
Coincidence sink-patient	10 (38%)	0 (0%)

this against the contamination of these siphons during this second period and the results were: rooms 1 and 18, again positive for OXA48 but rooms 12 and 20 were negative. The OR (room with OXA48 to room without OXA48: 10-8.5%) was 1.2; $p > 0.1$ non significant. In the Oncology hospitalization area (Fig. 4), we considered patients diagnosed between April and October 2013 (34 cases, 22 with strain ST11, and 10 with strain ST405) from 259 patients. This is similar to what occurs

throughout the hospital (strain ST11 is predominant). Four of the cases were clinical infections (three urinary tract infections and one septicemia). The rest were only colonization. In October the siphons were changed (because this did not require building work) and since then second period between October to Mars, with 155 studied patients), the number of OXA48 cases decreased (14); strain number 1 has been eliminated and only strain 11 remains, only as colonization.

Fig. 3. Spatial representation of OXA48-*K. pneumoniae* distribution by rooms in traumatology during two periods.

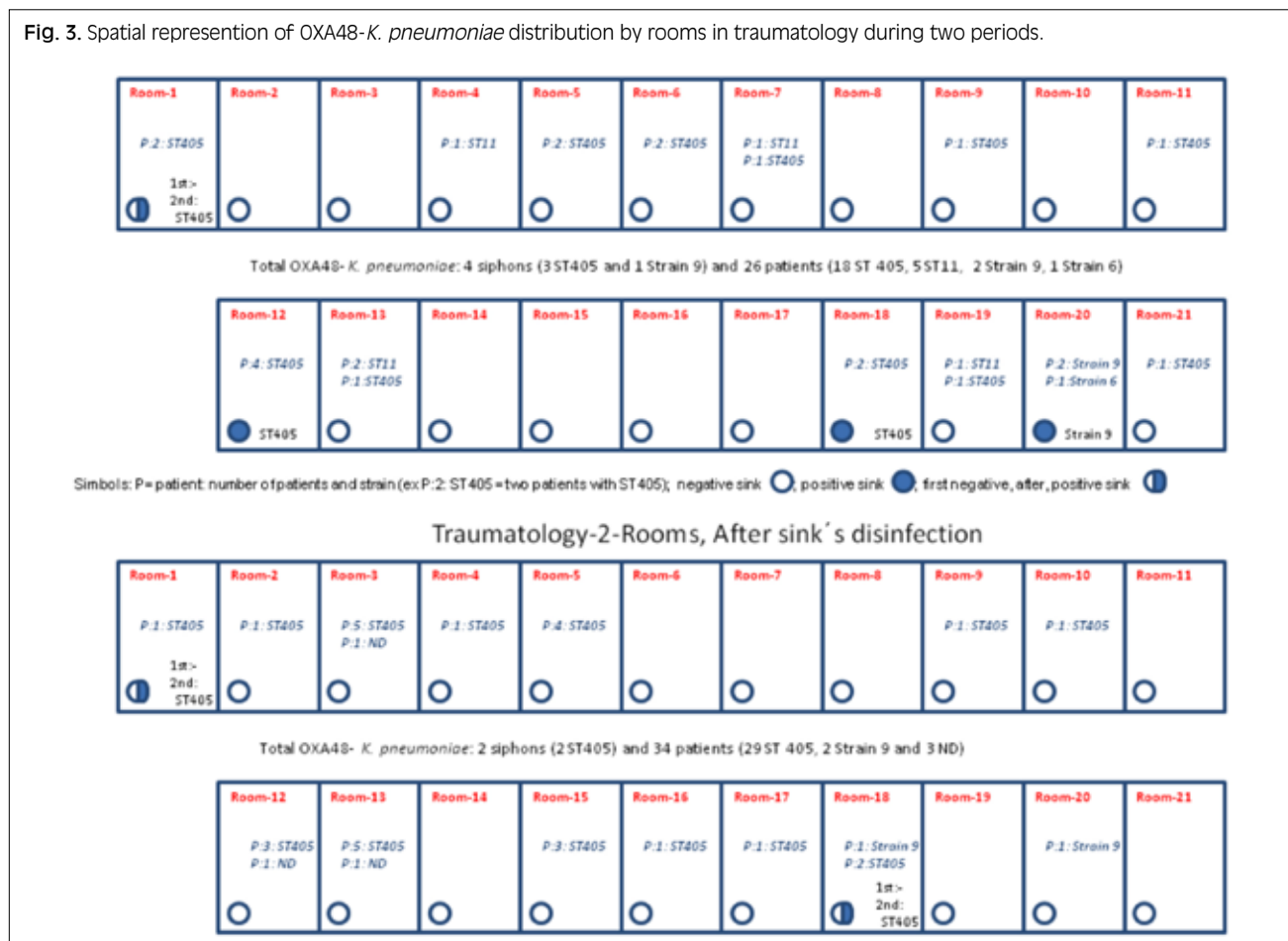
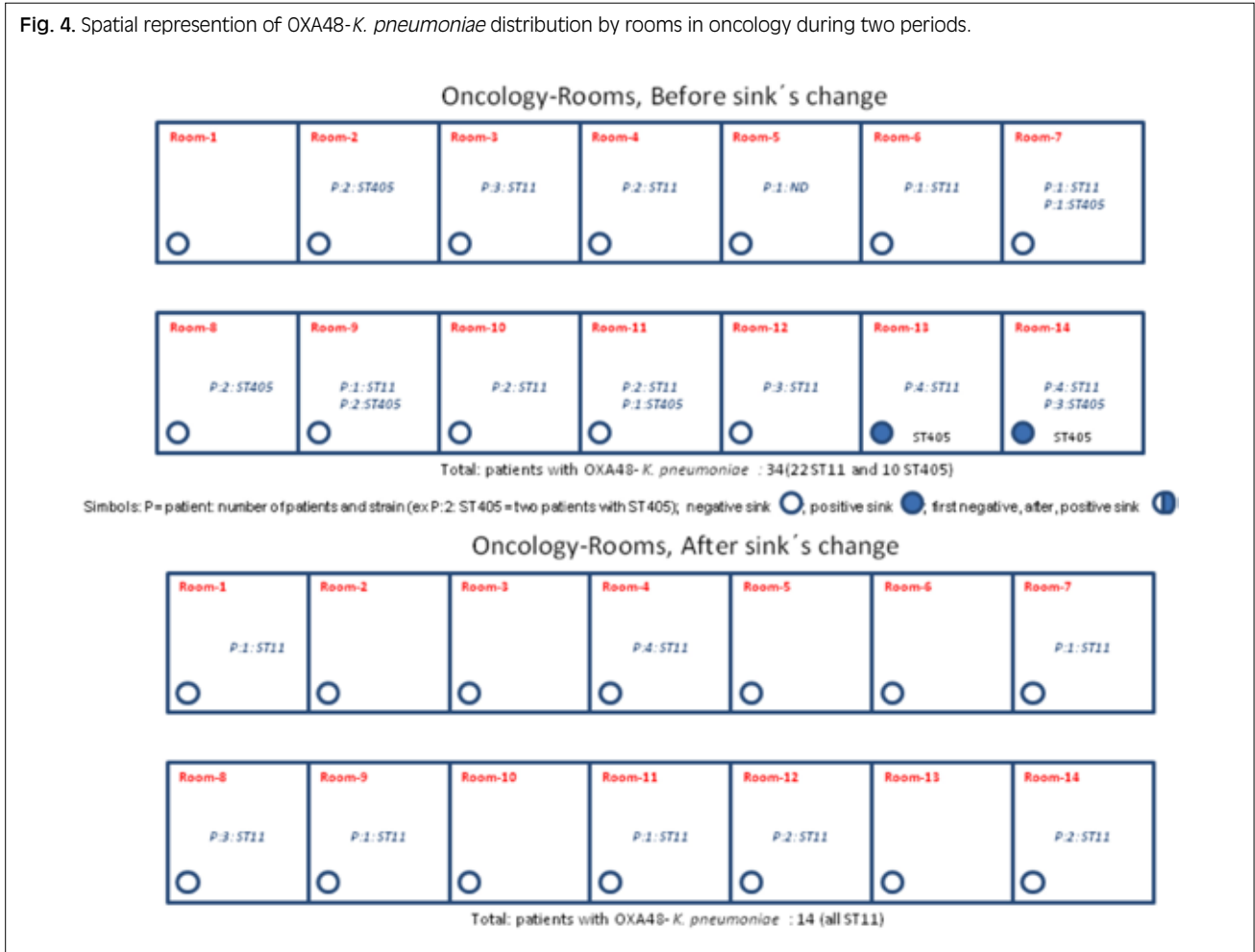


Fig. 4. Spatial representation of OXA48-*K. pneumoniae* distribution by rooms in oncology during two periods.



These cases predominated in rooms 13 and 14, where we detected OXA48-*K. pneumoniae* in the siphons. Before changing drains there were 28.2% in rooms 13 and 14, and 10.5% in the other rooms. The “rooms with OXA48 to room without these bacteria” OR was 3.3 (1.4-7.6; $p < 0.01$). After the change, the incidence dropped to 4.8% in rooms 13 and 14, and remained similar in the other rooms (9.7%). The relative risk in rooms with OXA/without OXA48 was 0.48 ($p > 0.1$). All these data suggest that OXA48 exposure from a contaminated sink is a probably interesting causal factor, and can be eliminated by changing the siphons without changing the other factors that continue to operate normally in the other rooms.

Lastly, the conjugation of OXA48-*K. pneumoniae* with other intestinal species of bacteria is not easy, because less than 0.5% of colonized patients by OXA-bacteria in our hospital, have two or more different species with OXA-plasmid, having been studied a grand number of rectal swabs in Oncology (2602) and Traumatology (2733) during the investigate period.

Discussion

PRINCIPAL FEATURES

1. High possibility of microorganism diffusion from contaminated hands, which continue to transmit a large number of microorganisms after more than 10 successive contacts to surfaces, but was more intense (higher number of FCU) in OXA than VIM bacteria. These are especially important for healthcare workers, but also for the patient and his/her family.
2. Microbial competition shows that VIM bacteria are inhibited by OXA organisms.
3. In another paper [22] we have demonstrated that these bacteria have a large capacity for survival on dry surfaces (same germ-carrier as used in experiments of diffusion from the hands): VIM and OXA48 bacteria can, respectively, survive for 35 and 21 days in the environment. *Klebsiella* with KPC-gene, has too a grand capacity of survival in environment [19, 23] and this can be greater in humid conditions [19, 24]. These facts can explain the concentration of cases of OXA48-*K. pneumoniae* in some rooms in Traumatology and Oncology, with a significant difference in risk between rooms with contaminated siphons by these bacteria and the other rooms. Risk was lowered after changing

(more reduction of risk) or disinfecting (heat plus chlorinated disinfectant, with worse results) the contaminated siphons.

However, the colonization of siphons by VIM bacteria was not related to human cases with similar microorganisms.

Normally, *Enterobacteriaceae* with carbapenemases are transmitted between patients by momentarily colonized hands of health personnel, family or patients, as reflected by the large numbers of surfaces contaminated by finger contamination (Fig. 2). But VIM-*K. pneumoniae* is less transmitted than OXA48. Perhaps the main reasons were the lower microbial adherence to surfaces or fingers by VIM-*K. pneumoniae* (after the first contact, the number of FCU was low) and it is not easily transferred between patients or from contaminated surfaces to patients, probably due to changes in the capsular polysaccharide (very frequent in *Klebsiella*) [25].

Moreover, based on the above obtained risk ratios, we believe that water-born OXA48 bacterial colonization may be another risk factor for patients admitted to these rooms, but not for VIM bacterial colonization with the exception of *K. oxytoca* with VIM, with cases described in other hospitals [2, 3] but not in these Traumatology patients, because we found 6 patients with this intestinal bacteria and 3 sink colonization, but no coincidence in strains between patients and their room. Perhaps, the less growth on the biofilm due to the lower capacity for microbiological competition between microorganisms with VIM versus those with OXA48 (together with the above commented minor adherence to skin or surfaces), reduces the likelihood of patient colonization, as was found in the Traumatology Service.

Contaminated siphons must be treated by pouring a large quantity of a disinfectant down the drain to remove the biofilm [19]. This may be successful in some sinks, but when the biofilm is thicker, it requires heat together with chemical treatment. However, when siphons can be changed (without requiring building work), it is more effective and easier to change them. Nevertheless, these changes must be made without patients in the room, as the water drops during the change are contaminated with OXA48 bacteria and, if not thoroughly cleaned and disinfected, the sprinkled surfaces may allow bacteria transmission to patients.

In other rooms of our hospital where OXA48 was detected in the sinks, we used heat plus 5 l of chlorinated disinfectant and, in all of them, the OXA48 bacteria were eliminated; we now indicate this double treatment when it is not possible to change the siphons. Moreover, 7-9 months after the siphon disinfection, 50% of the sinks have again become positive to OXA48. This indicates that a systematic disinfection can be a new measure in rooms with OXA48 in their siphons (e.g. every 6 months, because the cases detected in rooms 18 and 1 were at 6 and 8 months, respectively, after being made negative by siphon disinfection). In other paper [19], recontamination of sinks after change or disinfection of siphons are described, indicating that surveillance is necessary.

Finally we present our hypothesis (a possible explanation of the observations) about the relation of water pollution in the sinks and patient colonization. The slime begins to form a base inside the siphons. Bacteria that are discharged into the sink (water from washing bedridden patients or from the toilet of not bedridden patients) adhere to this base. The most common bacteria in siphon-biofilms are VIM. But if OXA48-bacteria reach the biofilm (especially *K. pneumoniae*, due to fimbriae 1 and 3 types [26]), they will out-compete the VIM bacteria and the successive bacterial layers formed in the biofilm will only carry OXA48 bacteria. When these reach a sufficient number, they can leave the siphon by the Venturi-effect, which occurs when you open the tap, and then contaminate directly the patient during toilet (hands, face, nose, eyes) or indirectly, through the cleaning cloths used on the sink, and after, these cloths contaminated surfaces of sink, bathroom, etc. These facts allow microorganisms to be carried to patients in the same room, given their high ability to survive on surfaces and easy transfer through contaminated hands, as demonstrated in our experiments. After to get OXA48-*K. pneumoniae* to nasal or oral cavities, these bacteria can transfer its bla-OXA-plasmid (due to its grand rate of conjugation [23]) toward *Klebsiella* endogenous, and, in few days, all digestive tract will be contaminated with OXA48-*K. pneumoniae*, allowing to be detected by rectal swab.

Conclusions

1. The discovery of OXA48-*K. pneumoniae* in the biofilm of sinks inside hospital rooms is a result of a colonized patient's stay, and the permanence of the bacteria, as a reservoir, may contribute to the perpetuation of an outbreak in hospital patients.
2. OXA48-*K. pneumoniae* grows better than VIM-*Enterobacteriaceae* on a biofilm (example, in the siphon of a sink).
3. After contamination of a patient, family or health worker's hand, they can distribute OXA48-*K. pneumoniae* to a large number of surfaces. In the case of VIM bacteria, the dissemination is less effective.
4. Heat-chemical treatment of this biofilm (repeated every 6 months) should be regarded as one step in the strategy for controlling an outbreak of OXA48-*K. pneumoniae*, if siphon change is not possible.
5. The elimination of VIM-bacteria from sink-reservoirs by disinfection is very difficult, but the risk for colonization of the patients admitted to these rooms is low.

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Author contributions

RH conceived and designed the research. GR, RH and MJV performed the microbiological analysis. LR and VP-B collected the epidemiological data. RH, MS and VP-B performed the statistical analysis. RH and MS wrote the manuscript. All authors revised and approved the final manuscript
Revision of the test by a native English speaker (C. Warren).

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