Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2019, 8(9): 12-20

Inhibition of Carbapenem-Resistant NDM-1 *Klebsiella pneumoniae* isolated from a Hospital Outbreak Patient by a Synbiotic: A Non-antibiotic Treatment Option

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ABSTRACT

Objective: *Klebsiella (K.) pneumoniae is globally responsible for an alarming increase in hospital infections, especially in intensive care units (ICUs). The acquisition of resistance against a broad range of antibiotics has turned infections with this pathogen into a major worldwide healthcare concern. The aim of the study was to investigate if multistrain synbiotics can complement the current treatment options of multidrug-resistant K. pneumoniae infections.* ***Methods:*** *Antimicrobial susceptibility and PCR testing were used to characterize the K. pneumoniae causing a hospital outbreak. Effect of multistrain synbiotic administration on the presence of K. pneumoniae in an infected patient was investigated by microbiological testing for the pathogen. Effects of the synbiotic mixture and its individual probiotic bacteria on K. pneumoniae isolated from patients and of the K. pneumoniae subsp. pneumoniae ATCC© 700603™ reference strain were investigated by pathogen in-vitro inhibition experiments.* ***Results:*** *The outbreak K. pneumoniae strain was found to be resistant against a range of antibiotics including carbapenems, and to be a producer of New Delhi metallo-β-lactamase 1 (NDM-1). Treatment of a NDM-1 K. pneumoniae carrier with a multistrain synbiotic resulted in successful elimination of the pathogen from the patient. In-vitro inhibition experiments showed that the NDM-1 K. pneumoniae (and the reference strain K. pneumoniae subsp. pneumoniae, ATCC© 700603™) could be effectively inhibited by the bacteria mixture of the synbiotic preparation.* ***Conclusion:*** *Findings of the study indicate for the first time that a multistrain synbiotic can add to the treatment repertoire available for the management of NDM-1 K. pneumoniae infections.*

Keywords: Hospital infection outbreak, Multidrug resistance, Pathogenic gram-negative bacteria, Probiotic bacteria, Extended-spectrum beta-lactamase

INTRODUCTION

Antibiotic resistance has become a major concern worldwide [1]. Extended-spectrum beta-lactamase (ESBL) producing, and carbapenem-resistant, Enterobacteriaceae, especially those of the species *K. pneumoniae*, are spreading at an alarming rate [2-7]. The steady trend of increasing resistance coupled with the lack of new antibiotics targeting resistant gram-negative bacteria is increasingly forcing clinicians to apply more aggressive antibiotic dosing regimens, such as prolonged administration and combination of different antibiotics [8-12]. Hospitals, and in particular the intensive care units (ICUs), are proliferation zones for multidrug-resistant gram-negative bacteria [13]. Reasons, among others, are the use of large amounts of broad-spectrum antibiotics and of invasive devices in ICUs, as well as the risk of cross-infection in severely ill patients [14-17].

K. pneumoniae is a gram-negative bacterium that is frequently isolated from samples collected from ICU patients. Carrier rates for *K. pneumoniae* in hospitalized patients are high (up to 77%) and seem to be related to the amount of administered antibiotics [18-20]. *K. pneumoniae* accounts for nearly 12% of all hospital-acquired pneumonia [18,20]. Being an opportunistic pathogen, *K. pneumoniae* primarily attacks immunocompromised patients who suffer from

severe underlying diseases. Patients with *K. pneumoniae* infections usually have a grim prognosis. The outlook is usually worse in diabetics, the elderly and those who are immunocompromised [21].

Carbapenems (e.g. imipenem or meropenem) represent the first-line therapy for severe infection by ESBL producing *K. pneumoniae* [22]. However, *K. pneumoniae* isolates resistant to carbapenems have been reported [23,24] and prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) has increased rapidly [25]. Infections with CRKP are associated with higher mortality compared to infections with carbapenem-sensitive *K. pneumoniae* (CSKP) and require appropriate initial antibiotic therapy [25,26]. There is no optimal treatment for CRKP. Treatment options include antibiotics from the polymyxin class, tigecycline, fosfomycin, aminoglycosides or dual therapy carbapenems. Combination therapy of 2 or more of the antibiotics may decrease mortality as compared to monotherapy alone [10,18].

As *K. pneumonia* is becoming resistant against more and more antibiotics, non-antibiotic strategies have to be considered for the management of infections with this pathogen. The fact that, firstly, *K. pneumoniae* can be present in the human body without causing disease and, secondly, carrier rates increase with the amount of administered antibiotics [19,20,27], hints at a fundamental role of balanced and diverse gut microbiota to keep *K. pneumoniae* under control in a healthy individual. Probiotics (products containing one or several probiotic bacterial strains) and synbiotics (products containing one or several probiotic bacterial strains and a prebiotic, e.g. fructo-oligosaccarides) have been discussed as alternative treatment or adjuvant therapy for a number of bacterial infections for which the use of antibiotics is either not recommended or emerging antibiotic resistance is of concern [1]. The present study investigates the potential of a complex multistrain synbiotic in the management of infections with multidrug-resistant *K. pneumoniae*.

MATERIALS AND METHODS

Sample Collection, Antimicrobial Susceptibility Testing, PCR Testing

Microbiological screening tests were performed in samples collected by mini-bronchoalveolar lavage (mini-BAL) from 2 patients (patient 1 and 2) and by anal swaps from 3 other patients (patients 3, 4 and 5) present in the ICU of the State Hospital of Jarocin, Poland. Antimicrobial susceptibility testing (AST) was performed according to a standard procedure (Kirby-Bauer method), established in the microbiology laboratory of the hospital. PCR testing for the presence of β-lactamases (NDM-1, KPC, and OXA-48) were performed by the Polish Reference Center for Antibiotic Sensitivity (KORLD) in Warsaw.

Antibiotic Therapies

All patients were initially treated empirically with antibiotics. Later, antibiotic therapy was guided by the results obtained from AST and PCR testing. Patients 1 and 2 were treated with large amounts of antibiotics. Respiration of both patients was supported by respirators.

Patients 3, 4 and 5 received significantly fewer antibiotics during their stay in the ICU, as they were inflammation-free carriers of the NDM-1 *K. pneumoniae* strain. None of these 3 patients were on respirators during their stay in the ICU.

Post-Outbreak Treatment of Patient 2 with a Multistrain Synbiotic Preparation

AST testing was performed during follow-up visits of patient 2 after her discharge from the hospital. The patient was treated for 30 days with a synbiotic preparation (once daily, administration before bedtime). Each capsule contained a mixture of Lactobacillus (Lb.) helveticus SP-27 (9.00×10^8 CFUs), Lactococcus (Lc.) lactis Ll-23 (9.00×10^8 CFUs), Lb. casei Lc-11 (2.25×10^8 CFUs), Lb. plantarum Lp-115 (2.25×10^8 CFUs), Lb. rhamnosus Lr-32 (4.50×10^8 CFUs), Bifidobacterium (B.) longum Bl-05 (6.75×10^8 CFUs), B. breve Bb-03 (4.50×10^8 CFUs), B. bifidum Bb-02 (2.25×10^8 CFUs), Streptococcus (St.) thermophilus St-21 (4.50×10^8 CFUs) and the prebiotic fructooligosaccharides (63 mg).

In-vitro Pathogen Inhibition

Antagonism between the synbiotic mixture or individual probiotic bacteria and the pathogenic bacteria NDM-1 *K. pneumoniae* isolated from patient 2 and the reference strain *K. pneumoniae* subsp. pneumoniae ATCC© 700603™) was marked by means of the bar graph method according to Strus [28,29]. Quantitative inhibition results of 3 independent experiments are presented as an arithmetic mean ± standard deviation (SD) of the inhibition zone.

For testing of the multistrain synbiotic mixture and of its individual probiotic strains, the particular bacteria suspensions of density 2 on the McFarland scale was sieved onto the MRS and incubated at 37ºC for 48 hours in the presence

of 6% CO_2. In the case of the individual bacteria strains, the suspensions were enriched with fructooligosaccharides (FOS) in a concentration of 63mg/ml.

After incubation in agar MRS, agar bars of 10 mm diameter were cut, which were transferred onto a medium that had previously been inoculated with the respective *K. pneumoniae* strain on the Mueller-Hinton medium. The inoculum of the bacterial strains studied was a suspension of the studied microbes in physiological salt of density 2, according to the McFarland scale. After placing the bar with the studied culture of the medium with both studied microbes, they were placed at a temperature of 4ºC for 4 hours. Further incubation of the medium was conducted at a temperature of 37ºC for 24 hours without limiting oxygen access. After incubation, the diameter of the growth inhibition zone around bars containing the studied strains were marked/calculated in mm, and the result was given together with the diameter of the bar itself.

Bacterial Strains and Synbiotic Preparation

NDM-1 *K. pneumoniae* was cloned and cultivated from a sample taken from patient 2 of the outbreak. The reference strain *K. pneumoniae subsp. pneumoniae* ATCC© 700603™ [30] was purchased from MicroBioLogics Inc., St. Cloud, MN 56303, USA. All individual bacterial strains in the synbiotic mixture (B. bifidum Bb-02 [31-34]; Lb. plantarum Lp-115 [33,35-43]; Lb. casei Lc-11 [44]; B. breve Bb-03, Lc. lactis Ll-23, Lb. rhamnosus Lr-32 [33,39,45-47], St. thermophilus St-21, Lb. helveticus SP-27, B. longum Bl-05 [39,48,49]) are commercially available and were provided by Bifodan A/S, Bogbinderivej 6, 3390 Hundested, Denmark. The multistrain synbiotic preparation is commercially available as MULTILAC® SYNBIOTIKUM and was provided by Vivatrex GmbH, Martinstr. 10-12, 52062 Aachen, Germany.

RESULTS

NDM-1 *K. pneumoniae* Outbreak

On 20th November 2017, routine microbiological testing of 2 ICU patients revealed the presence of *K. pneumoniae* bacteria. An identical broad pattern of antibiotic resistance against β-lactam antibiotics (including carbapenems), ciprofloxacin, sulfamethoxazole-trimethoprim and colistin (polymyxin E) for *K. pneumoniae* from both samples was found. The isolated *K. pneumoniae* was susceptible to gentamycin and medium-susceptible to amikacin. Table 1 provides an overview of the resistance profile of the *K. pneumoniae* strain isolated from patient 2, in comparison to that of the ESBL producing reference strain *K. pneumoniae* subsp. pneumoniae ATCC© 700603™.

Table 1 Antibiotic resistance profiles of the *K. pneumoniae* isolated from outbreak patient 2 and of the ESBL-producing reference strain *K. pneumoniae subsp. pneumoniae* ATCC© 700603™

Antibiotic class	Antibiotic	*K. pneumoniae subsp. pneumoniae* ATCC© 700603™	NDM-1 *K. pneumoniae* (isolate from patient 2*)
Penicillin	Ampicillin	R	-
	Piperacillin	R	-
Penicillin with beta-lactamase inhibitor	Amoxicillin/clavulanate	S	R
	Piperacillin/tazobactam	S	R
Cephalosporin	Cefuroxime	R	R
	Cefotaxime	R	R
	Ceftazidime	-	R
Carbapenem	Imipenem	S	R
	Meropenem	S	R
Quinolone	Ciprofloxacin	S	R
Aminoglycoside	Gentamycin	MS	S
	Amikacin	S	MS
Other	Sulfamethoxazole-trimethoprim (biseptol)	R	R
	Colistin (polymyxin E)	-	R

R: Resistant, S: Susceptible, MS: Medium Susceptible, "-": Not Tested; *AST performed with *K. pneumoniae* isolated and cloned from a sample taken from patient 1 showed identical results

Samples from all 5 patients present in the ICU were taken and PCR testing by KORLD confirmed the presence of New Delhi metallo-β-lactamase 1 (NDM-1) in all samples. PCR testing for the KPC and OXA-48 type β-lactamase subtypes revealed the absence of these β-lactamase subtypes.

A hospital outbreak was declared on 27th November 2017 and the hospital infection control team implemented a comprehensive and complex outbreak management procedure. Due to the severity of the outbreak it was finally decided to close the ICU for intensive decontamination. Key information about the patients involved in the *K. pneumoniae* outbreak is provided in Table 2.

Table 2 Key information about patients involved in the NDM-1 *K. pneumoniae* outbreak in the State Hospital of Jarocin, Poland

Patient	Sex	Age	Reason for admission to ICU	Days in ICU	Antimicrobial susceptibility testing[1]	PCR testing[2,3]	Status at discharge from ICU
1	Male	71	Stroke, respiratory failure	68	ESBL incl. carbapenem resistance	MBL (NDM-1) positive	Deceased
2	Female	53	Generalized inflammation, respiratory failure	80	ESBL incl. carbapenem resistance	MBL (NDM-1) positive	Alive
3	Female	74	After surgery	39	-	MBL (NDM-1) positive	Alive
4	Female	50	After surgery	19	-	MBL (NDM-1) positive	Alive
5	Male	55	Head injury, due to car accident	16	-	MBL (NDM-1) positive	Alive

ESBL: Extended-Spectrum Beta-Lactamase, MBL: Metallo Beta Lactamase, NDM-1: New Delhi Metallo-Beta Lactamase-1; 1: Samples tested were taken from patients 1 and 2 by mini-BAL, patients 3, 4 and 5 were inflammation symptom-free carriers and no AST was performed; 2 Samples tested were taken from patients 1 and 2 by mini-BAL and from patients 3, 4 and 5 by anal swaps; 3: Samples tested were negative for the presence of KPC and OXA-48 types of beta-lactamases

The 5 patients from the outbreak can be divided into 2 groups. Patients of the first group (patients 1 and 2) stayed the longest in the ICU (68 and 80 days, respectively), were treated with large amounts of antibiotics and showed extensive inflammation of bacterial etiology. Despite intensive treatment, the condition of patient 1 gradually deteriorated and he died after 68 days in the ICU. Patient 2 recovered gradually and was transferred to the surgical ward of the hospital after 80 days in the ICU. She stayed an additional 31 days in the surgical ward, before being discharged from the hospital. ASTs performed at the time of discharge and during later follow-up visits revealed that she was still a carrier of *K. pneumoniae*. Patients of the second group (patients 3, 4 and 5) stayed on average 25 days (range 16-39 days) in the ICU and received significantly fewer antibiotics, as they were inflammation-free carriers of the NDM-1 *K. pneumoniae* strain. Table 3 provides an overview about the total (accumulated) amounts of antibiotics given to the different patients during their stay in the ICU.

Table 3 Total (accumulated) amounts of antibiotics administered to the patients from the *K. pneumoniae* outbreak during their stay in the ICU

			Patient				
Antibiotic Class	**Antibiotic**	**Units**	**1**	**2**	**3**	**4**	**5**
Penicillin with ß-lactamase inhibitor	Amoxicillin/Clavulanate	g	-	-	-	50.4	-
	Pipieracillin/Tazobactam	g	306	234	-	-	--
Cephalosporin	Cefuroxime	g	-	-	63	-	--
Carbapenem	Imipenem	g	24	36	-	-	-
Quinolone	Ciprofloxacin	g	5.2	2.4	-	-	-
	Levofloxacin	g	-	-	10.5	10.5	10.5
Amino-glycoside	Gentamycin	g	4.3	0.5	-	-	-
	Vancomycin	g	51	-	-	-	-
	Amikacin	g	-	1.5	-	-	-
Other	Sulfamethoxazole-trimethoprim (biseptol)	g	-	1	-	-	-
	Colistin (polymyxin B)	mio IU	96	24	-	-	-

Post-Outbreak Treatment of Patient 2 with a Synbiotic Multistrain Preparation

At the time of discharge from the hospital, patient 2 was an inflammation-free carrier of *K. pneumoniae*. Presence of the pathogen was repeatedly confirmed by testing of samples taken by anal swaps during follow-up visits of the patient. Due to the ongoing presence of *K. pneumoniae*, treatment with a commercially available multistrain synbiotic was initiated. After 30 days of therapy, microbiological testing of a sample taken by anal swap revealed the absence of *K. pneumoniae*.

***In-vitro* Pathogen Inhibition**

In-vitro pathogen inhibition experiments revealed that growth of the NDM-1 *K. pneumoniae* strain isolated from patient 2 was inhibited by the synbiotic mixture given to the patient post-outbreak. Growth of this *K. pneumoniae* strain was also inhibited, however, to a lesser extent, by the individual probiotic strains of the mixture. The inhibitory effect of the individual strains varied significantly, with those of *St. thermophilus* St-21 and *Lb. helveticus* SP-27 being the strongest and those of *Lc. lactis* Li-23 and *Lb. rhamnosus* Lr-32 being the weakest. Similar inhibitions were observed when the ESBL-producing reference strain *K. pneumoniae* subsp. pneumoniae ATCC© 700603™ was investigated (Figure 1).

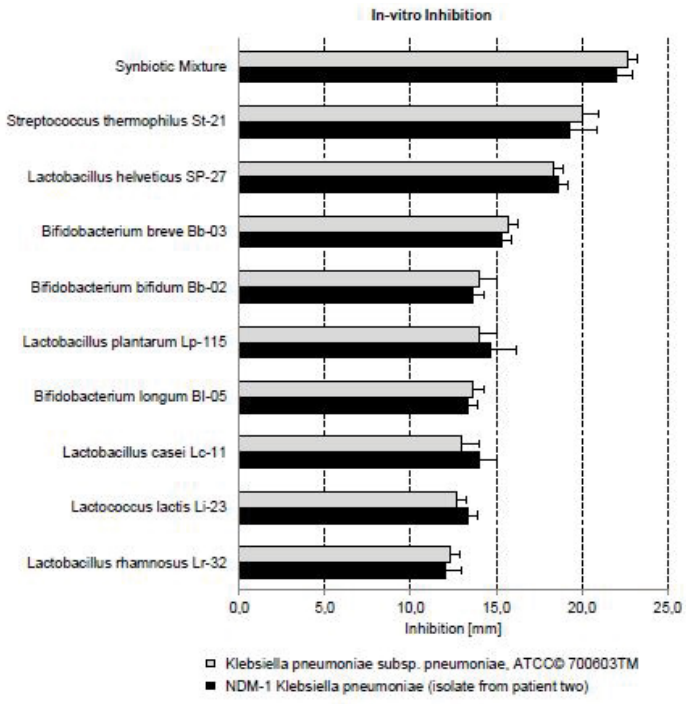

Figure 1 *In-vitro* inhibition of NDM-1 *K. pneumoniae* isolated from patient 2 and of *K. pneumoniae* subsp. ATCC© 700603™ by the synbiotic mixture and its individual probiotic constituents

DISCUSSION

Microbiological characterisation of samples taken from the 5 patients of the outbreak leads to the conclusion that all patients became infected by the same NDM-1-producing *K. pneumoniae* strain. As no microbiological screening of patients before the admission to the ICU was performed, the origin of the NDM-1 *K. pneumoniae* strain causing the outbreak could not be determined.

Outbreak patients 3, 4 and 5 were carriers of NDM-1 *K. pneumoniae* but never exhibited signs of inflammation. Antibiotic therapy of these patients was moderate. In contrast, patients 1 and 2 were treated extensively with antibiotics, showed strong inflammation of bacterial etiology and stayed a long time in the ICU. While most of the antibiotics administered to patients 1 and 2 had no effect on the multidrug-resistant NDM-1 K. pneumoniae, they definitely extinguished a good part of the gut-microbiotas of the two patients. This disturbance of the gut microbiota might well have eliminated or at least weakened its capability to prevent or limit the colonisation of the patients' guts

by the pathogenic *K. pneumoniae*. In addition, a disturbed gut microbiota is linked to a malfunction of the immune system [50-53], a circumstance which might also have contributed negatively to the conditions of these two patients. These observations support the hypothesis that extensive usage of broad-spectrum antibiotics is a risk factor for bad outcomes in patients infected by multi-drug resistant *K. pneumoniae* strains [16,54]. A conservative usage of antibiotics, at least until information from AST has become available, should be considered as a measure to lower the risk of an outbreak. Antibiotic therapy should be guided by a good understanding of the pathogen causing the infection and should be accompanied by the administration of probiotic bacteria to support a balanced and diverse gut-microbiota.

Post outbreak treatment of patient 2, being a symptom-free carrier of *K. pneumoniae*, with a multistrain synbiotic resulted in the elimination of the *K. pneumoniae* pathogen. As we were only able to treat this one patient, no general conclusions about the clinical effectiveness of the administered synbiotic product can be made. However, the observed effect in this patient encourages further studies aiming to investigate the effect of synbiotics on *K. pneumoniae* proliferation.

Few studies have investigated the effect of individual Lactobacillus and Bifidobacteria strains on the *in-vitro* growth of *K. pneumoniae* [55,56]. None of these studies investigated the effects on ESBL-producing or NDM-1 *K. pneumoniae*. The present study shows that both NDM-1- and an ESBL-producing *K. pneumoniae* strain can be effectively inhibited *in-vitro*, and potentially also *in-vivo*, by a complex multistrain synbiotic mixture. No significant difference in the inhibition of the two investigated *K. pneumoniae* strains was found, indicating that the different resistance profiles of the two strains had no relevance for the inhibition by the synbiotic mixture. The inhibitory effect of the synbiotic mixture was found to be stronger than that of its individual constituents. Similar results have been shown by our group for the inhibition of *Salmonella typhimurium* by this synbiotic preparation and its constituents [57]. A reason for the superior effect of the mixture might be synergistic effects among the individual probiotic bacteria, leading to a stronger overall inhibitory effect on pathogenic bacteria [58-60].

CONCLUSION

K. pneumoniae and its multidrug-resistant variants can be inhibited by multistrain synbiotics suggesting that synbiotics can play a positive role in the management of patients infected with this bacterial pathogen. The potential contribution of synbiotics in clinical practice should be further investigated.

DECLARATIONS

Acknowledgment

We thank the team from KORLD in Warsaw for performing the PCR testing, L. Sommer Mikkelsen and K. Bundvig Barken of Bifodan A/S for providing the individual probiotic strains used in the *in-vitro* inhibition experiments, S. Hanna from Cambridge Assessment English for proof-reading, English style editing, and useful suggestions.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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