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Phenotypical and molecular assessment of the virulence potential of KPC-3-producing *Klebsiella pneumoniae* ST392 clinical isolates

Danilo D'Apolito[a,b], Fabio Arena[c,d], Viola Conte[e], Lucia Henrici De Angelis[e], Giuseppina Di Mento[b], Anna Paola Carreca[a], Nicola Cuscino[b], Giovanna Russelli[b], Gioacchin Iannolo[b], Floriana Barbera[b], Salvatore Pasqua[a], Francesco Monaco[b], Francesca Cardinale[b], Gian Maria Rossolini[f,g], Pier Giulio Conaldi[b], Bruno Douradinha[a,b,*]

[a] *Fondazione Ri.MED, Palermo, Italy*
[b] *IRCCS-ISMETT, Palermo, Italy*
[c] *Dipartimento di Medicina Clinica e Sperimentale, Università di Foggia, Foggia, Italy*
[d] *IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy*
[e] *Dipartimento di Biotecnologie Mediche, Università degli Studi di Siena, Siena, Italy*
[f] *Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Firenze, Florence, Italy*
[g] *SOD Microbiologia e Virologia, Azienda Ospedaliera Universitaria Careggi Florence, Italy*

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ABSTRACT

Klebsiella pneumoniae is a Gram-negative bacterium of clinical importance, due to its resistance to several antibiotic classes. We have identified 4 clinical isolates of *K. pneumoniae* sequence type (ST) 392 KPC-3-producing strains from patients at the Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione (IRCCS-ISMETT), a Southern Italian transplantation health facility, during a routine surveillance for carbapenemase-producing *Enterobacterales* from in-house clinical samples. Since those were among, to the best of our knowledge, the first KPC-producing *K. pneumoniae* ST392 isolated in Europe, we assessed their virulence potential, to understand if this particular ST can become an endemic clinical threat. ST392 isolates were investigated to assess their virulence potential, namely resistance to human sera, formation of abiotic biofilms, adhesion to biotic surfaces, exopolysaccharide production and in vivo pathogenesis in the wax moth *Galleria mellonella* animal model. ST392-belonging strains were highly resistant to human sera. These strains also have a high capacity to form abiotic biofilms and high levels of adhesion to the human epithelial colorectal adenocarcinoma HT-29 cell line. An increase of transcriptional levels of genes involved in serum resistance (*aroE* and *traT*) and adhesion (*pgaA*) was observed when compared with the *Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain ATCC 700603 reference strain. Infection of *G. mellonella* larvae with ST392 clinical isolates showed that the latter were not highly pathogenic in this model. Together, our results indicate that ST392 isolates have the potential to become a strain of clinical relevance, especially in health settings where patients are immunosuppressed, e.g., transplant recipients.

1. Introduction

In the last years, an increase of bacterial nosocomial infections has been observed, due to their plastic ability to acquire multiple antibiotic resistances, including to carbapenems, a last resort class of antibiotics. Several bacterial species such as Gram-positive methicillin-resistant *Staphylococcus aureus*, *Clostridioides* (*Clostridium*) difficile, and vancomycin-resistant *Enterococcus* and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* are now considered of clinical relevance, especially in contexts where patients are immunosuppressed, e.g., transplant recipients. Multidrug resistant pathogens not only reflect themselves in a huge spectrum of associated, diverse pathologies, contributing to the increase of morbidity and mortality of hospital patients but are also an economical burden for healthcare facilities (Dalton et al., 2020; Morris and Cerceo, 2020). Currently, many efforts have focused in the development of novel therapies against multidrug resistant pathogens, such as vaccines or new drugs, although unfortunately without tangible

* Corresponding author at: Fondazione Ri.MED c/o IRCCS-ISMETT, Via Ernesto Tricomi 5, Palermo, 90127, Italy.
E-mail address: bdouradinha@fondazionerimed.com (B. Douradinha).

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success (Boucher et al., 2017; Buchy et al., 2020).

K. pneumoniae is a Gram-negative, encapsulated, nonmotile bacterium of clinical importance. This bacterium readily colonizes mucosal surfaces and, from there, gain access to other tissues and establish severe infections, such as pneumonia, sepsis, urinary tract infections and liver abscesses (Paczosa and Mecsas, 2016). Resistance to several antibiotics has been reported, reducing the number of effective treatments. Initially, only immunosuppressed patients and people who must spend a medium to long-term recovery period in health facilities were considered of particular concern, due to the ability of this pathogen of colonizing medical devices and instruments, such as catheters, etc. (Paczosa and Mecsas, 2016). However, the worldwide emergence of multidrug resistant *K. pneumoniae* strains and the detection of hypervirulent strains for which the current available interventions scarcely work against make healthy individuals also susceptible to infection by this bacterial pathogen and consequent pathologies. Thus, *K. pneumoniae* is increasingly becoming a public health concern (Paczosa and Mecsas, 2016). In fact, the World Health Organization has issued a list of antibiotic resistant bacteria in which the need of novel interventions against *K. pneumoniae* is considered crucial (WHO, 2017).

Historically, *K. pneumoniae* strains have been classified based on their capsular type (K). Categorization was done through an ELISA based assay but most laboratories lacked the specific antibodies to perform such serological assay. Also, this technique could not efficiently predict the K-type of many clinical strains, which express a non-previously identified K-type for which there are no available antibodies, or the K-type of non-capsulated strains (Pan et al., 2015; Wyres et al., 2016). To circumvent this problem, *K. pneumoniae* strains are typically classified by their sequence types (STs), which are defined by their nucleotide sequences at 7 loci (*mdh*, *infB*, *tonB*, *gapA*, *phoE*, *pgi*, and *rpoB*) (Chen et al., 2014b; Paczosa and Mecsas, 2016). Bioinformatics tools online (e.g., Institut Pasteur *Klebsiella* MLST at https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef) allow the classification of the *K. pneumoniae* strain based on the sequence of all the genes aforementioned. Also, classification can be done based on the diversity of the capsule loci (KL) genetic synthesis region. The KL-types are defined based on the sequence of genes that encode the core capsule biosynthesis machinery (*galF*, *wzi*, *wza*, *wzb*, *wzc*, *gnd* and *ugd*) (Pan et al., 2015; Wyres et al., 2016). As for ST determination, the same bioinformatics tool online may be used to identify KL-types based on the sequence of these genes.

Genetic analysis of Italian carbapenem resistant *K. pneumoniae* clinical isolates showed an epidemiological prevalence of ST512 clade II and ST258, with a recent emergence of ST307 and ST101 isolates (Arena et al., 2020; Conte et al., 2016; Giani et al., 2013; Villa et al., 2017). However, many other STs are identified in nosocomial infections, increasing the risk of occurrence of new, more aggressive clones. The early detection of such clinical isolates and their respective ST is crucial to adopt adequate and efficient countermeasures against the infections caused by this bacterium. Recently, we have identified the first KPC-3 –producing ST392 clinical isolate worldwide (from hereafter referred as ST392-2), from a patient with urinary tract infection, in Palermo, Italy (Di Mento et al., 2017). Whole genome sequencing of this isolate showed the presence of genes encoding for proteins associated with virulence and adhesion to surfaces, such as fimbriae type 1 (*fimA* and *fimH*) and 3 (*mrkA* and *mrkD*), *ompA*, *pal*, *lppA*, iron ABC transporter periplasmic iron-binding protein and putative allantoin permeases. The presence of genes involved in resistance, such as bla_{KPC-3}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{TEM-1}, *aac(6′)Ib*-cr, *aac(3)-IIa*, *aph(3′)-Ib* and *qnrB1*, was also confirmed in the ST392-2 clinical isolate. Mutations in the *gyrB1*, *parC* and *parE* genes implied in resistance to antibiotics were also detected. Resistance to aminoglycosides (gentamicin, tobramycin), β-lactams (amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftazidime, cefuroxime, ertapenem, imipenem, meropenem, piperacillin/tazobactam), fluoroquinolones (ciprofloxacin, levofloxacin), fosfomycin and trimethoprim/sulfamethoxazole was observed. On the other hand, ST392-2 isolate was susceptible to amikacin and colistin. Later, we have identified other three ST392 clinical isolates, from bile, blood and from a central venous catheter. Despite different origins, they all shared the same virulence and resistance genes, suggesting they are representatives of the same strain. Interestingly, KPC-3 producing ST392 isolates displaying resistance to colistin have later been observed in another area of Southern Italy (Esposito et al., 2018). Due to the capacity of this particular ST to acquire easily resistance to several antibiotics, we decided to further characterize phenotypically and at virulence level the ST392 isolates to understand if they have the potential to become high-risk multidrug resistant *K. pneumoniae* clones of clinical relevance.

Table 1
K. pneumoniae clinical isolates used in this work.

Isolate	Year	ST	KL	Gender	Isolation site	Accession Number
ST512-1	2017	512	154	F	Wound swab	SAMN09813396
ST512-2	2017	512	154	M	Urine	SAMN09813397
ST512-3	2017	512	154	M	Sputum	SAMN09813395
ST307-1	2017	307	173	M	Blood	SAMN09813400
ST307-2	2016	307	173	M	Rectal swab	SAMN09813398
ST307-3	2017	307	173	M	Blood	SAMN09813399
ST307-4	2016	307	173	M	Blood	SAMN09813401
ST101-1	2016	101	137	M	Blood	SAMN09813402
ST392-1	2016	392	187	M	Bile	SAMN09813403
ST392-2	2017	392	187	F	Urine	SAMN09813404
ST392-3	2015	392	187	F	Central Venous Catheter	SAMN09813406
ST392-4	2016	392	187	M	Blood	SAMN09813405

2. Material and methods

2.1. K. pneumoniae strains and reagents

In this work, we used *K. pneumoniae* strains isolated at IRCCS-ISMETT (Palermo, Italy) from several sources (Table 1) and the carbapenem susceptible *Klebsiella quasipneumoniae* subsp. *similipneumoniae* reference strain ATCC 700603 (ATCC, Manassas, VA, USA), formerly known as *K. pneumoniae* K6 (Elliott et al., 2016) and hereafter referred as reference strain ATCC 700603. To ensure that the clinical isolates do not lose their resistance phenotype, they were always grown in the presence of meropenem, final concentration 2 μg/mL, while the reference strain was grown without antibiotics. All reagents used in this work were from Sigma-Aldrich (Darmstadt, Germany) unless specified otherwise.

2.2. Clinical isolates identification and ST and minimal inhibitory concentration determination

Identification of *K. pneumoniae* clinical isolates was confirmed and their ST and capsular locus (KL) determined by next generation sequencing (NGS) (Table 1) as previously described (Di Mento et al., 2017; Gona et al., 2017; Monaco et al., 2018). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers illustrated in Table 1. Minimal inhibitory concentrations (MIC) were determined (Tables 2, S1 and S2) according to the latest release of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints, as detailed elsewhere (Di Mento et al., 2017; Gona et al., 2017; Monaco et al., 2018).

2.3. Serum resistance

Serum resistance assays were performed by mixing 100 μl of *K. pneumoniae* isolates grown overnight in bacterial MacConkey liquid

Table 2
Minimal inhibitory concentrations (MIC[a]; mg/L) of *K. pneumoniae* ST392 ISMETT clinical isolates.

Antibiotic	*K. pneumoniae* ST392 ISMETT clinical isolates			
	#1	#2[b]	#3	#4
Aminoglycosides				
Amikacin	S < 4.0	I 4.0	S 1.0	I 4.0
Gentamicin	R 4.0	R 16.0	R 16.0	R 16.0
Tobramicin	R > 4.0	ND[c]	R > 4.0	ND
β-lactamases				
Amoxicillin/ Acid Clavulanic	R 32.0	R 32.0	R 32.0	R 32.0
Cefepime	R 8.0	R 8.0	R 8.0	R 64.0
Cefotaxime	R 4.0	R 64.0	R 64.0	R 64.0
Ceftazidime	R 8.0	R 64.0	R 64.0	R 64.0
Cefuroxime	R 8.0	ND	R 8.0	ND
Ertapenem	R 1.0	ND	R 8.0	ND
Imipenem	R 8.0	R 16.0	R 8.0	R 16.0
Meropenem	R 16.0	R 16.0	I 8.0	R 16.0
Piperacillin/ Tazobactam	R 16.0	R 128.0	R 32.0	R 128.0
Fluoroquinolones				
Ciprofloxacin	R 1.0	R 4.0	R 4.0	R 4.0
Levofloxacin	R 2.0	ND	R 2.0	ND
Other				
Colistin	S 1.0	S 0.5	S 0.5	S 0.5
Fosfomycin	R 64.0	R 256.0	S 16.0	R 128.0
Trimethoprim/ Sulfamethoxazole	R 4.0	R 320.0	R 320.0	R 320.0

[a] Level of resistance according to EUCAST guidelines: R, resistant; I, intermediate; S, sensitive.
[b] Published previously in Di Mento et al J Hosp Infection 2018.
[c] ND, not done.

culture diluted to a final concentration of 2.5×10^7 cells/mL with a pool of 300 µl of fresh, non-heated human sera obtained from three healthy volunteers (Villa et al., 2017). The bacteria/serum mixture was incubated at 37 °C and 250 rpm and samples corresponding to time point zero (T0) and after 30, 60 and 120 min were collected, serially diluted and plated on MacConkey agar and incubated overnight at 37 °C prior to viable cell counts. The assays were repeated three times using three different pools of sera obtained from different volunteers.

2.4. Abiotic biofilm production assay

K. pneumoniae strains were grown overnight at 37 °C and diluted to an optical density 600 nm (OD_{600}) of 0.1, of which 20 µl were added to 180 µl MacConkey broth supplemented with 1% glucose and incubated for 18 h in a 96-well flat-bottomed plastic tissue culture plate (Vuotto et al., 2017). As a negative control, MacConkey broth supplemented with 1% glucose alone was used, while the reference strain ATCC 700603 was selected as positive control. After incubation, the plates were washed with PBS three times, followed by drying 1 h at 60 °C and staining with 180 µl of 2.3 % crystal violet solution for 15 min. The crystal violet attached to the adherent bacteria was solubilized with 180 µl of 33 % glacial acetic acid and the absorbance was read at OD_{570}. As stated elsewhere (Vuotto et al., 2017), the OD cut-off (ODc) is defined as three standard deviations above the mean OD of the negative control and the strains were into the following categories: nonbiofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2 ODc), moderate biofilm producers (2ODc < OD ≤ 4 ODc) and strong biofilm producers (4 ODc < OD). Experiments were repeated three times.

2.5. Adhesion assay

To measure the adherence potential of *K. pneumoniae* strains, they were incubated with differentiated human colon adenocarcinoma cell line HT-29 (Istituto Zooprofilattico Sperimentale di Brescia, Brescia, Italy) in 6-well culture plates. The cells were cultured in RPMI 1640 (Euroclone SpA, Pero MI, Italy) containing 10 % fetal bovine serum (FBS; Euroclone SpA) without antibiotics in an atmosphere of 5% CO2 at 37 °C. Confluent monolayers of HT-29 cells used were obtained after 15 days of culture. Per well, 1 mL RPMI of a suspension of 10^8 bacteria per ml, obtained by overnight growth at 37 °C in liquid Luria-Bertani (LB) broth, was added to the culture medium. Cell growth was monitored as the optical density at 600 nm (OD_{600}). Prior to the adhesion test, bacteria were rinsed three times with PBS and resuspended in the appropriate volume of RPMI containing 2% D-mannose. Cells were incubated for 1 h at 37 °C and 5% CO_2 (Di Martino et al., 1996). The wells were washed there times with PBS, and the cells were lysed by 0,5% Triton X-100 and plated for CFU determination. Cells incubated with RPMI alone were used as negative control. Serial 8-fold dilutions were prepared in PBS and plated onto LB and incubated at 37 °C to quantify adhered bacteria (Favre-Bonte et al., 1999). Adhesion experiments were repeated three times.

2.6. Quantitative real time PCR

Transcriptional levels of *K. pneumoniae* genes related to serum resistance (*aroE*, *htrA*, *pal*, *lpaA*, *rfaH* and *traT*) (Doorduijn et al., 2016; Hennequin and Robin, 2016) and adhesion (*mrkA* and *pgaA*) (Vuotto et al., 2017) were assessed by quantitative real time PCR to confirm which genes might have a role in the serum resistance and adhesion ability demonstrated by ST392 clinical isolates. Briefly, bacteria were grown in MacConkey broth overnight, diluted to an OD_{600} of 0.2 and allowed to grow for 4 h. Bacteria in the exponential phase were collected and RNA extracted with an RNeasy™ Mini Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. Subsequently, RNA was converted into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Milano, Italy), in agreement with the protocol supplied by the producing company, and quantitative real time PCR was done using 50 ng of cDNA as template and the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher), as per the supplier's instructions. Expression levels were calculated with the 2ΔΔCt method (Livak and Schmittgen, 2001). Expression of bacterial specific sequences was normalized with that of a house-keeping gene, *rho* (Gomes et al., 2018). Primers sequences used can be observed in Table S3.

2.7. String and Congo red agar tests

Both tests were performed as described elsewhere (Vuotto et al., 2017). For the string test, *K. pneumoniae* isolates were grown on MacConkey agar plates and touched with an inoculation loop. Strains were classified as hypermucoviscous if a string longer than 5 mm was observed, otherwise strains were classified as mucoid. For the Congo red agar test, *K. pneumoniae* strains were incubated overnight at 37 °C in Brain Heart Infusion agar plates supplemented with 5% sucrose and 0.08 % Congo red. Red colonies displaying a dry crystalline phenotype were classified as good exopolysaccharides producers and white or pink colonies as weak or moderate exopolysaccharides producers, respectively.

2.8. Galleria mellonella larvae infection model

Bacteria were grown in Luria-Bertani broth, harvested during exponential phase, and washed once with PBS. Bacteria were diluted in PBS to approximately 1×10^5 CFU/mL. TruLarv™ *G. mellonella* larvae (Biosystems Technology, Exeter, UK) were injected with 10 µL of bacterial suspension, containing approximately 1×10^3 CFU/larva, or with PBS (negative control). Infection with *K. pneumoniae* NTUH-K2044 strain (Fang et al., 2004) and with *K. pneumoniae* KKBO-1 strain

(Cannatelli et al., 2013) were used as virulence positive and negative control, respectively. Larvae were placed in petri dishes with food and kept in the dark at 37 °C for 3 days and checked daily for mortality assessment (Insua et al., 2013). At least three independent experiments were performed.

2.9. Statistics

Statistical analyses were executed with GraphPad Prism 8.3 software (GraphPad Software, La Jolla, CA, USA). Results are shown as Student's paired, two-tailed t test, and considered significant when the *p* values obtained yielded values inferior to 0.05 (*), to 0.01 (**), or to 0.001 (***).

2.10. Ethical clearance

Informed consent of all in-house patients was obtained according to internal regulations before procedures were performed on their clinical samples.

3. Results

3.1. Identification of KPC-3-producing K. pneumoniae ST392 clinical isolates

We have recently identified the first KPC-3-producing *K. pneumoniae* ST392 clinical isolate, present in a urinary tract infection (Di Mento et al., 2017). A retrospective analysis of carbapenem-resistant *K. pneumoniae* clinical isolates from ISMETT from 2011 to 2017 showed that, in recent years, three additional KPC-3-producing *K. pneumoniae* ST392 isolates from different patients were present in the collection (Table 1). These isolates were from blood, bile and from a central venous catheter. Sequencing showed that these isolates possessed > 99.9 % identity, suggesting they derive from the same clone. The three clinical isolates displayed a similar resistance phenotype (Table 2) that was also similar to that reported previously for ST392-2 (Di Mento et al., 2017). Discrepancies between strains regarding their resistance behavior were observed for amikacin and fosfomycin (Table 2). As for ST392-2, genetic sequences corresponding to virulence factors like fimbriae type 1 (*fimA* and *fimH*) and 3 (*mrkA* and *mrkD*), *ompA*, *pal*, *lppA*, iron ABC transporter periplasmic iron-binding protein and putative allantoin permeases were identified in the remaining ST392 isolates. Likewise, resistance genes such as bla_{KPC-3}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{TEM-1}, *aac(6′)Ib*-cr, *aac(3)-IIa*, *aph(3′)-Ib* and *qnrB1*, were also present in the ST392 clinical isolates, as well as mutations in the *gyrB1*, *parC* and *parE* genes, which are involved in antibiotic resistance.

3.2. KPC-3-producing K. pneumoniae ST392 clinical isolates are able to survive in human serum

Serum resistance and survival is a virulence factor used by Gram-negative bacteria, allowing their spreading throughout the organism and sustaining further infections. Thus, we assessed the ability of KPC-3-producing *K. pneumoniae* ST392 isolates to resist serum-mediated killing (Fig. 1). After 2 h incubation with non-inactivated human serum, these strains showed a high level of survival, which was statistically significant when compared to reference strain ATCC 700603 (Fig. 1). Also, the levels of survival of all strains increased gradually during the chosen time points (data not shown). When matched to other *K. pneumoniae* strains of clinically relevant STs (Table 1), we observed resistance to serum was similar to the one displayed by ST512 and ST101 isolates, and higher than the one shown by ST307 isolates (Fig. 1). Thus, the resistance to human serum displayed by ST392 isolates is similar or even higher than that reported by strains belonging to STs of clinical importance.

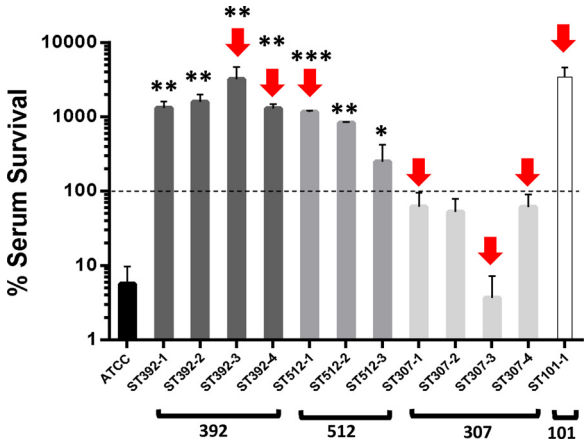

Fig. 1. Resistance of clinical isolates to serum after a 2 h incubation. Clinical isolates, unlike reference strain ATCC 700603, are able to resist serum complement mediated killing, except for isolate ST307-3. Also, most of the strains multiply on these conditions, except for isolates belonging to ST307. Red arrows indicate strains which were isolated from infection situations, indicating there is no difference between clinical isolates from infection sites and from colonization situations. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3.3. KPC-3-producing K. pneumoniae ST392 clinical isolates are strong abiotic biofilm producers

Some *K. pneumoniae* strains have the ability to form biofilms in abiotic surfaces. This is particularly relevant when such biofilms occur in medical devices, such as catheters or other prosthetic devices, which may lead to spreading and persistence of healthcare-associated infections (Paczosa and Mecsas, 2016; Vuotto et al., 2017, 2014). We quantified the biofilm formation ability and observed that all four isolates were strong biofilm producers (Fig. 2). Concomitantly, we observed that reference strain ATCC 700603 and ST307 clinical isolates were also strong biofilm producers while ST512 and ST101 clinical isolates were weak biofilm producers (Fig. 2).

3.4. KPC-3-producing K. pneumoniae ST392 clinical isolates adhere to the HT-29 epithelial intestinal cell line

K. pneumoniae strains are able to colonize mucosal surfaces, from where they can access the bloodstream and other organs, causing infections. We assessed the adhesion levels of our clinical isolates to an epithelial intestinal cell line, HT-29, previously differentiated for 15

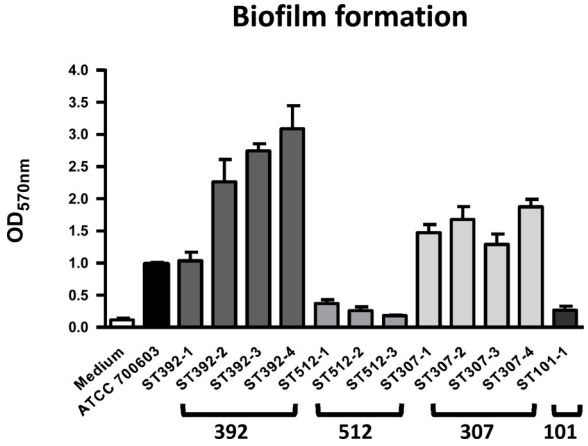

Fig. 2. Abiotic biofilm formation capacity of *K. pneumoniae* clinical isolates. Reference strain ATCC 700603 and isolates belonging to ST392 and ST307 are strong biofilm producers. On the other hand, isolates belonging to ST512 and ST101 are weak biofilm producers.

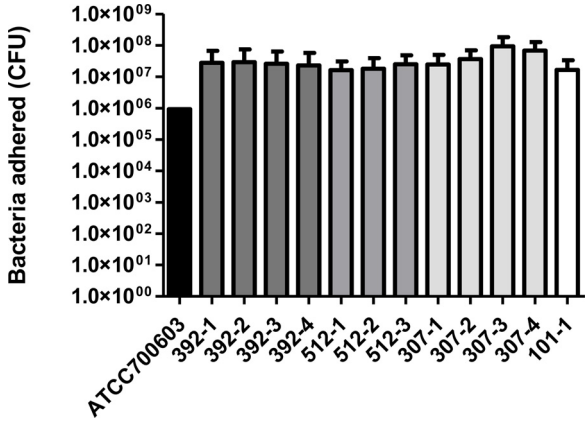

Fig. 3. Adhesion of *K. pneumoniae* clinical isolates to differentiated HT-29 cells. These clinical isolates show a very high adhesion level to these epithelial intestinal cell line when compared to the reference strain ATCC 700603, although not statistically significant.

days prior to co-incubation with bacteria. We observed that all clinical isolates, including the ST392 isolates, showed a high level of adhesion, when compared to the reference strain ATCC 700603 (Fig. 3), although the difference was not statistically significant within strains.

3.5. KPC-3-producing K. pneumoniae ST392 clinical isolates show upregulation of genes aroE, traT and pgaA

Transcriptional levels of genes related to serum resistance (*aroE*, *htrA*, *pal*, *lpaA*, *rfaH* and *traT*) and adhesion (*mrkA* and *pgaA*), as suggested elsewhere (Doorduijn et al., 2016; Hennequin and Robin, 2016; Vuotto et al., 2017), were assessed to confirm which genes might have a role in the serum resistance and adhesion ability demonstrated by ST392 clinical isolates. Our results show a significant increase in the transcriptional levels of *aroE* and *traT* genes when matched to those of the reference strain ATCC 700603 (Fig. 4), suggesting that expression of the related proteins may contribute to the serum resistance observed. Likewise, the increase in the levels of *pgaA* might influence the levels of adhesion shown by the ST392 isolates (Fig. 4). The levels of the other tested genes did not show major differences between ST392 isolates and the reference strain ATCC 700603 (data not shown). A significant increase of *aroE*, *traT* and *pgaA* genes was also observed in the other tested clinical isolates (Fig. 4).

3.6. KPC-3-producing K. pneumoniae ST392 showed moderate production of exopolysaccharides

High production of exopolysaccharides might correlate with high levels of biofilm production (Vuotto et al., 2017). Using the Congo Red agar test, reference strain ATCC 700603 and ST392 and ST307 clinical isolates showed moderate production of exopolysaccharides (pink colonies), in agreement with their high capacity to form biofilms (Table 3). In agreement, the ST101 isolate displayed a poor production of exopolysaccharides (white colonies), which correlates to its low ability to form biofilms. Interestingly, although ST512 clinical isolates did not show an aptitude to form biofilms, they exhibited high levels of exopolysaccharides production (red colonies), probably due to their membrane gluco-configuration and to the presence of amino acids in their cell wall (Vuotto et al., 2017) and not related with their biofilm formation capacity.

Table 3
Production of exopolysaccharides and mucoid phenotype of KPC3-producing *K. pneumoniae* clinical isolates.

Strain	Production Exopolysaccharides		String Test
ST392-1	pink	moderate production	mucoid
ST392-2	pink	moderate production	mucoid
ST392-3	pink	moderate production	hypermucoviscous
ST392-4	pink	moderate production	mucoid
ST512-1	red	good production	mucoid
ST512-2	red	good production	mucoid
ST512-3	red	good production	mucoid
ST307-1	pink	moderate production	mucoid
ST307-2	pink	moderate production	mucoid
ST307-3	pink	moderate production	mucoid
ST307-4	pink	moderate production	mucoid
ST101-1	white	poor production	mucoid
ATCC 700603	pink	moderate production	mucoid

3.7. KPC-3-producing K. pneumoniae ST392-3 strain displayed a hypermucoviscous phenotype

To assess the mucoid phenotyping, we used the string test (Vuotto et al., 2017). All strains showed a mucoid phenotype except for ST392-3 (Table 3), which displayed a string longer than 5 mm, thus being classified as hypermucoviscous, a characteristic shared among *K. pneumoniae* hypervirulent strains.

3.8. KPC-3-producing K. pneumoniae ST392 isolates exhibited a low pathogenic potential in a G. mellonella larvae infection model

Pathogenicity of ST392 clinical isolates was assessed by infecting wax moth *G. mellonella* larvae with 10^3 CFU/larva and assessing viability at 24, 48 and 72 h post-infection. As expected, infection with NTUH-K2044 strain and with KKBO-1 strain led to high and low levels of mortality, respectively (Fig. 5). ST392 isolates showed virulence levels similar or inferior to the KKBO-1 strain, suggesting that these isolates are scarcely pathogenic (Fig. 5). Remaining clinical isolates belonging to ST512, ST307 and ST101 and reference strain ATCC

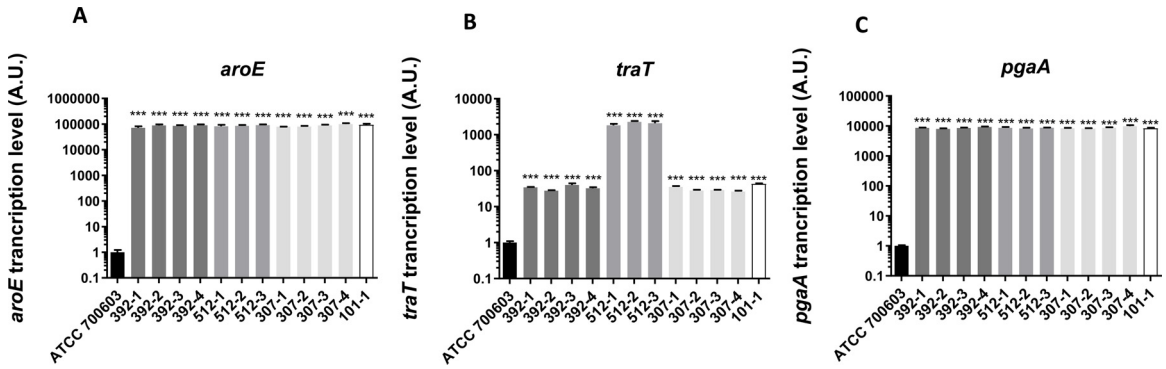

Fig. 4. Transcriptional levels of *aroE*, *traT* and *pgaA* genes in *K. pneumoniae* clinical isolates. When compared to reference strain ATCC 700603, transcriptional levels of all 3 genes show that they are upregulated in the clinical isolates. ***$p < 0.001$.

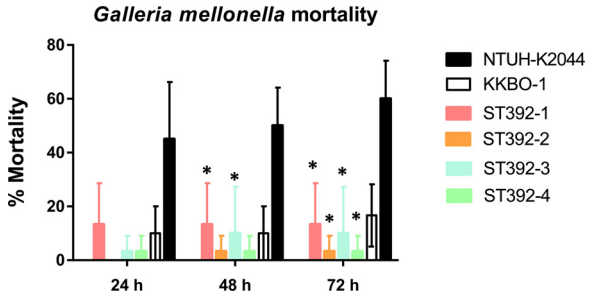

Fig. 5. Pathogenicity of *K. pneumoniae* ST392 clinical isolates in *G. mellonella* larvae at 24, 48 and 72 h. When compared to the virulent strain NTUH-K2044, the ST392 isolates are scarcely pathogenic in this model. *$p < 0.05$.

700603 also demonstrated lower levels of pathogenicity in respect to the NTUH-K2044 strain, using this model (data not shown). These results are in accordance with the fact that multidrug resistant and virulent *K. pneumoniae* populations are generally non overlapping (Bialek-Davenet et al., 2014).

4. Discussion

In this work, we have assessed the virulence potential of KPC-3-producing *K. pneumoniae* ST392 clinical isolates. Nosocomial *K. pneumoniae* ST392 isolates have been isolated in several countries. Non KPC-producing *K. pneumoniae* ST392 clinical isolates have been reported in Italy (Fasciana et al., 2019), Colombia (Rojas et al., 2016), Spain (Oteo et al., 2013), Iran (Kiaei et al., 2019) and in an outbreak in a Mexican tertiary-care hospital (Bocanegra-Ibarias et al., 2017). On the other hand, KPC2-producing *K. pneumoniae* ST392 have been identified in China (Yang et al., 2013), USA (Gomez-Simmonds et al., 2015) and Argentina (Cejas et al., 2019) while KPC-3-producing strains were isolated in Italy (Di Mento et al., 2017; Esposito et al., 2018; Fasciana et al., 2019). Interestingly, both KPC2- and KPC-3-producing *K. pneumoniae* ST392 isolates were observed in a study in USA (Macesic et al., 2020). Thus, *K. pneumoniae* ST392 isolates seem to be widely spread worldwide.

The KPC-3-producing ST392 isolates from IRCCS-ISMETT were resistant to multiple antibiotics (Table 2) and it is noteworthy to mention that the most recent isolate, ST392-2, in 2017, showed higher overall levels of resistance when compared with the remaining ST392 isolates, except for cefepime (Table 2). Interestingly, KPC-3-producing *K. pneumoniae* ST392 isolates isolated also in Palermo, albeit in different health facilities, showed a similar antibiotic resistance profile (Fasciana et al., 2019). Similarly to ours, their isolates carried resistance genes $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, *aph(3')-Ib*, *gyrA/B* and *parC/E* and *mrk* virulence genes. They also displayed resistance genes $bla_{SHV\text{-}69}$ and $bla_{OXA\text{-}1}$ while our isolates showed a different SHV isoform, $bla_{SHV\text{-}11}$, and no bla_{OXA} resistance gene. Also, while all ST392 Palermo isolates were susceptible to colistin (Table 2; (Fasciana et al., 2019)), a KPC-3-producing ST392 clinical strain isolated in Naples, Italy, with mutations in the genes *crrB* and *mgrB* was reported as colistin resistant (Esposito et al., 2018). This indicates how ST392 isolates are developing resistance to multiple antibiotics in rapid and diverse ways.

Resistance to human sera is a strategy which allows Gram-negative bacteria to efficiently spread throughout the organism and supporting further infections. KPC-3-producing *K. pneumoniae* ST392 clinical isolates demonstrated a high level of resistance to human sera (Fig. 1), comparable or even higher than that showed by ST512, ST307 and ST101 isolates. It has been previously shown that ST512, ST307 and ST101 isolates have a high resistance to human sera (Villa et al., 2017). Thus, this strengthens the possibility that KPC-3-producing *K. pneumoniae* ST392 clinical isolates, due to their ability to survive human serum complement factors, can also spread through the blood and cause sepsis and systemic infections of clinical importance. Our results show that transcription of *aroE* and *traT* genes was significantly upregulated in our clinical isolates, including ST392 isolates. *aroE* gene is involved in aromatic amino acids synthesis and its depletion leads to an increased serum susceptibility (Doorduijn et al., 2016). Plasmids carrying the $bla_{CTX\text{-}M\text{-}15}$ resistance gene usually confer higher resistance to human serum, supposedly due to the copresence of the *traT* gene (Hennequin and Robin, 2016; Robin et al., 2012). Thus, we believe that these two genes participate in the serum resistance displayed by our isolates. Also, our preliminary results showed a deposition of complement membrane attack complex (MAC, C5b-9, data not shown), which should lead to bacterial lysis and subsequent death. Since we observe a MAC deposition but also resistance to serum, this probably indicates that the capsule of our isolates prevents the MAC to reach the outer membrane of the bacteria through steric hindrance, as suggested elsewhere (Doorduijn et al., 2016).

Biofilm formation is an important feature for *K. pneumoniae*, since it increases the ability of this bacterium to resist antibiotics and human sera complement, and to colonize mucosal surfaces (Vuotto et al., 2017, 2014). We observed that all KPC-3-producing *K. pneumoniae* ST392 clinical isolates were strong biofilm producers (Fig. 2) and showed strong levels of adhesion to the HT-29 epithelial intestinal cell line (Fig. 3), in agreement with their resistance to several antibiotics (Table 2) and high levels of resistance to human serum (Fig. 1). Together, this demonstrates that KPC-3-producing *K. pneumoniae* ST392 clinical isolates may persist in abiotic material, e.g., surgical material or catheters, or colonize mucosal surfaces and, from there, spread further causing successful systemic infections, due to their ability to survive in human sera and their resistance to several antibiotics classes. The *pgaABCD* system in *K. pneumoniae* is responsible for the envelope-spanning Pga machinery for the synthesis and secretion of poly-β-linked N-acetylglucosamine (PNAG), having an active role in biofilm formation and mouse intestinal colonization (Chen et al., 2014a). In all our isolates, we observed an increase of transcriptional levels of the *pgaA* gene, suggesting this gene is actively participating in both processes, biofilm formation and cellular adhesion. On the other hand, transcriptional levels of *mrkA* did not differ from the ATCC 700603 control strain (data not shown). This is in agreement with previous findings showing that not all *K. pneumoniae* biofilm-forming strains produce type 3 (MR/K) fimbriae (Vuotto et al., 2014). Our KPC-3-producing *K. pneumoniae* ST307 clinical isolates displayed a similar phenotype to ST392 isolates, regarding biofilm formation (Fig. 2), cellular adhesion (Fig. 3) and *pgaA* transcriptional levels (Fig. 4). Interestingly, our ST512 and ST101 were weak biofilm producers (Fig. 2), despite showing increased resistance to antibiotics (Table S1) and to human sera (Fig. 1) and high *pgaA* transcriptional levels (Fig. 4), suggesting these strains possess other strategies that compensate their inability to form biofilms.

We have also characterized these strains regarding their exopolysaccharide production and mucoviscosity phenotypes. Exopolysaccharidic matrix would act as an adhesin and contribute to biofilm formation (Vuotto et al., 2017). In agreement with their ability to form biofilms, the reference strain ATCC 700603 and the ST392 and ST307 isolates displayed a moderate exopolysaccharide production phenotype (Fig. 2 and Table 3). Also in agreement with its poor ability to form biofilms, ST101 isolate showed a weak production of exopolysaccharides (Fig. 2 and Table 3). Conversely, ST512 isolates had high levels of exopolysaccharides production (Table 3) although they are poor biofilm producers (Fig. 2). This discrepancy observed for ST512 isolates may be due to independent interactions of the stain agent used, Congo Red, with either amino acids present in their wall or different configuration of sugars in their membrane (Vuotto et al., 2017). All tested isolates showed a mucoid phenotype (Table 3), except for ST392-3, which was hypermucoviscous (Table 3). Hypermucoviscosity is usually observed in hypervirulent strains and confers them protection against serum complement killing and phagocytosis by neutrophils (Kumabe and Kenzaka, 2014; Vuotto et al., 2017). This suggests that at least one of the ST392 isolates might be evolving to a more

hypervirulent phenotype.

The larval stage of the wax moth *G. mellonella* has been proved to be a useful model of *K. pneumoniae* infection and allows to discriminate between highly virulent and less pathogenic strains of this bacterium (Insua et al., 2013). Infection of these larvae with 10^3 CFU of each of the ST393 isolates lead to mortality rates of 15 % or lower after 72 h (Fig. 5). This is in agreement with another independent study, which showed that, in the *G. mellonella* model, the lethal dose 50 (LD_{50}) and 90 (LD_{90}) of a KPC-3-producing ST392 *K. pneumoniae* clinical isolate were 7.24×10^5 and 8.27×10^6, respectively (Esposito et al., 2018). It has been observed that strains possessing antimicrobial resistance genes and associated with nosocomial infections show variability in their pathogenicity during *G. mellonella* infection (Diago-Navarro et al., 2014) and usually do not display a hypervirulent phenotype (Bialek-Davenet et al., 2014). Thus, the pathogenic potential of the KPC-3-producing *K. pneumoniae* ST392 isolates appears to rely in their diverse antimicrobial resistance capability and not in an intrinsic hypervirulent phenotype.

In this study, we have characterized the virulence potential of KPC-3-producing *K. pneumoniae* ST392 isolates. We and others have shown that these strains have a high genome plasticity, possessing several virulence genes and are able to acquire multidrug resistance genes (Di Mento et al., 2017; Esposito et al., 2018; Fasciana et al., 2019; Macesic et al., 2020). Their ability to form abiotic biofilms and perform efficient cellular adhesion enhances their clinical importance, since it allows these bacteria to infect new hosts through medical devices and to persist, until conditions become favorable for spreading into further infections. Once in the bloodstream, the ability of the KPC-3-producing *K. pneumoniae* ST392 isolates to survive human serum and to resist to multiple antibiotics helps them to establish in and infect different organs, leading to diverse pathologies. We believe that KPC-3-producing *K. pneumoniae* ST392 isolates are an emergent ST of clinical relevance and further epidemiological studies will help to understand how these strains are spreading worldwide as well as their nosocomial importance.

CRediT authorship contribution statement

Danilo D'Apolito: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation. **Fabio Arena:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **Viola Conte:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **Lucia Henrici De Angelis:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Giuseppina Di Mento:** Data curation, Investigation. **Anna Paola Carreca:** Data curation, Investigation. **Nicola Cuscino:** Data curation, Formal analysis, Investigation, Methodology. **Giovanna Russelli:** Data curation, Investigation. **Gioacchin Iannolo:** Conceptualization. **Floriana Barbera:** Data curation, Investigation, Methodology. **Salvatore Pasqua:** Data curation, Investigation. **Francesco Monaco:** Data curation, Investigation, Methodology, Resources. **Francesca Cardinale:** Data curation, Methodology, Resources. **Gian Maria Rossolini:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. **Pier Giulio Conaldi:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision. **Bruno Douradinha:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126551.

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