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Full Length Research Paper

Antimicrobial susceptibility profile of *Enterococcus* species isolated from cows with clinical mastitis and from bulk milk tanks in Brazil

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Vancomycin-resistant enterococci (VRE) have been reported as a major hazard to human and animal health, causing problems like clinical mastitis in dairy cattle and nosocomial infections in humans. The aim of this study was to determine the *in vitro* microbial susceptibility profile of enterococci isolated from dairy farms in São Paulo and Minas Gerais states, Brazil. A total of 310 samples of *Enterococcus* species from bovine mastitis (52) and bulk tanks (258) were analyzed. The sensibility profile was studied by the disk diffusion method, and the isolates intermediately resistant to vancomycin were submitted to the minimum inhibitory concentration (MIC) test. The enterococci also were classified according to their multiresistance profile. All isolates were sensitive to vancomycin. Most of the isolates from the bulk tanks were resistant to cephalexin (93.8%), novobiocin (98.8%), cefoxitin (91.9%) and oxacillin (91.9%), while those isolated from mastitis presented a high resistance to trimethoprim/sulfamethoxazole (84.7%), novobiocin (100%), cefoxitin (88.5%) and oxacillin (80.8%). All isolates were sensitive to vancomycin (VSE). The high prevalence of isolates resistant to multiple antimicrobials emphasizes the risks existing in the use of ineffective antibiotics.

Key words: Enterococci, mastitis, multiresistence, milk, vancomycin.

INTRODUCTION

Enterococci are Gram-positive cocci and opportunistic pathogens frequently isolated from hospital environments (Arias and Murray, 2012; Prieto et al., 2016). These bacteria are considered highly multi-drug resistant, with

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special regards to vancomycin (Raza et al., 2018), indicating an increased risk of nosocomial infections in humans (De Kraker et al., 2013; Raza et al. 2018). Glycopeptide antibiotics (e.g. vancomycin) are the main drugs used against Gram-positive bacteria causing severe human infections unresponsive to other antibiotics. Indeed, glycopeptides provide an alternative therapy against bacteria such as methicillin- or oxacillin-resistant *Staphylococcus aureus* (MRSA and ORS, respectively). However, some enterococci isolates have been described as resistant to glycopeptides, which can be an obstacle for the control and treatment of enterococci infections (Tavares, 2014). A study developed in Estonia observed two *Enterococcus faecalis* isolates from humans with *vanB* genes highly resistant to vancomycin. These molecularly characterized resistance genes are present in strains with diverse origins, suggesting the occurrence of plasmid transfer events (Aun et al., 2021).

Although enterococci are considered sensitive to vancomycin, a rise in strains with resistance genes of potential transmission has been recorded (Tavares, 2014; Raza et al., 2018). These strains are termed Vancomycin-Resistant Enterococci (VRE) and are considered 'superbacteria' as determined by genotypic and phenotypic features (Werner, 2012; Tavares, 2014).

The importance of determining these genotypic and phenotypic profiles lies in the understanding of the movement of resistance genes, a phenomenon produced by plasmids and transposons in which genes are transferred across enterococci and other Gram-positive bacteria (Aun et al., 2021; Van Schaik et al., 2010; Werner, 2012).

Moreover, enterococci present relevance as an etiological agent of bovine mastitis. In a recent study performed in Poland with 2,000 mastitic milk samples from confirmed cases of bovine mastitis, 21.3% were caused by *Enterococcus* species (Różańska et al., 2019). Such inflammatory disease represents a major issue in dairy farming, especially when caused by VREs, which are more difficult to treat and can represent a huge risk of dissemination of resistance genes to other pathogens that causes mastitis, like *S. aureus* (Werner, 2012). Besides, VRE-induced mastitis can be a risk to public health as a foodborne pathogen, since VREs may be present in subclinical cases of mastitis and, therefore, infect humans through consumption of contaminated milk from apparently healthy cows (Werner, 2012; Kateete et al., 2013).

In Brazil, the most studied and prevalent bacterium in cattle is *S. aureus*. A recent study analyzed 400 strains isolated from mastitic animals in four Brazilian states, and showed that these strains have important resistance genes in almost all classes of antimicrobials currently used for human and animal treatment in the country (Pérez et al., 2020). In the case of *Klebiseila pneumoniae*, another very prevalent bacterium in the country, the presence of genes encoding β-lactamases was observed in several bacteria, including genes for drug resistance for commonly used drugs in the treatment of mastitis in Brazilian dairy herds (gentamicin, cephalosporins, sulfamethoxazole-trimethoprim, tetracycline), as well as antimicrobials of critical importance to human health (meropenem, ceftazidime, fluoroquinolones) (Nobrega et al., 2021). A recent study, observed the species diversity and antimicrobial resistance patterns of *Enterococcus* spp. isolated from mastitis cases, milking machine and dairy cow environment. As a result, of the 365 isolates studied from several Brazilian municipalities, 1.9, 0.3 and 0.6% were resistant to penicillin, vancomycin and teicoplanin, respectively. This is a recent data that demonstrates the current situation (Juliano et al., 2022).

The relevance in assessing the multiresistance of enterococci isolated from bovine milk is in four main premises: (1) human transmission through ingestion of contaminated milk, which has paramount importance in Brazil where 20 to 30% of milk and dairy products are sold without inspection or heat treatment (IBGE, 2015); (2) significant gaps are found in the literature with respect to the multiresistant profile of enterococci isolated from bovine milk in Brazil; (3) *Enterococcus* spp. are neglected etiological agents of cattle mastitis (Kateete et al., 2013); and (4) *Enterococcus* spp. can transmit resistance genes to *S. aureus*, the most important pathogen of mastitis in cattle (Werner, 2012).

Therefore, the aims of this study were to determine the *in vitro* antimicrobial susceptibility profile of *Enterococcus* spp. isolated from bulk tanks and mastitic milk samples from dairy farms in the states of São Paulo and Minas Gerais (Brazil).

MATERIALS AND METHODS

The farm

A total of 310 species of *Enterococcus* spp. were studied, of which 52 were from confirmed cases of bovine clinical mastitis and 258 from bulk tanks. The milk samples positive for enterococci were obtained between 2018 and 2019 from four dairy farms in São Paulo State and six in Minas Gerais State, Brazil.

All ten cattle herds had mastitis control programs, including computer software for data record, a somatic cell count (SCC) in bulk tanks up to 400,000 cs mL^{-1}, a minimum of 200 lactating cows, and an automatic milking machine. In addition, the milk from the dairy farms was supplied by a high-production Holstein Friesian cattle (> 20 L each animal per day).

Animals with mastitis were treated with different classes of antibiotics, depending on the farm of origin. The most used antibiotics, in descending order, were: ceftiofur (third generation cephalosporin), tetracycline hydrochloride (tetracycline class), neomycin (aminoglycoside class), bacitracin (aminoglycoside class), cefquinoma (cephalosporin class), cefoperazone (cephamycins class) and enrofloxacin (quinolones class).

Isolation

The cases of clinical mastitis were based upon detection of macroscopic changes common to mastitic milk (e.g. clumps, pus or blood) and the systemic signs of mastitis in dairy cows (e.g. fever, loss of appetite or behavior changes) (Radostits et al., 2007). Mastitic milk samples from these animals were collected in sterile vials following teat disinfection with 70% alcohol and were immediately kept refrigerated (maximum of 7°C) during transfer to the Research Nucleus on Mastitis (NUPEMAS) laboratory of the São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu, São Paulo State, Brazil. At the same time, monthly bulk tanks milk samples of each farm were transferred to the laboratory with the same sterility and refrigeration conditions.

In the laboratory, mastitic milk samples were spread on MacConkey agar and blood agar plates, with the latter being supplemented with 5% defibrinated ovine blood. Samples were incubated at 37°C under aerobic conditions, and examined every 24 h for three days. The bacteria were classified according to the criteria adopted by the National Mastitis Council (NMC, 1999). The colonies that were morphologically compatible with enterococci were analyzed after Gram stain by microscopy, as well as by catalase and hemolysins tests. The samples were assayed by the following biochemical tests as described by Facklam and Collins (1989) and updated by Facklam (2007): growth in halophyte broth (6.5% NaCl), esculin hydrolysis, hydrolysis of L-pyrrolidonyl-beta-naphthylamide (PYR), arginine decarboxylase test, pigment production, motility, tetrazolium reduction, mannitol, arabinose, raffinose and sorbitol fermentation test.

From the bulk tanks, 1 mL of sample was pipetted into test tubes along with 9 mL of 0.1% sterile peptone water (CLSI, 2014). Later, dilutions were carried out in a serial fashion from 10^{-1} to 10^{-3}. A total volume of 1 mL from each dilution was spread onto Enterococcosel agar, and incubated at 37°C for 24 h to identify the enterococci colonies based on their morphological characteristics.

***In vitro* antimicrobial susceptibility profile**

As proposed by Bauer et al. (1966), the antimicrobial susceptibility was evaluated by the disk diffusion method, where the bacteria were submitted to the following antimicrobials in Mueller-Hinton agar: Ampicillin - 10 µg (AMP), Cephalexin - 30 µg (CL), Cefoxitin - 10 µg (CFO), Ciprofloxacin - 10 µg (CIP), Enrofloxacin - 10 µg (ENR), Gentamicin -10 µg (GEN), Marbofloxacin - 5 µg (MRB), Neomycin - 30 µg (N), Novobiocin - 5 µg (NOV), Oxacillin - 10 µg (OXA), Penicillin G - 10 U.I. (PEN), Sulfamethoxazole/Trimethoprim - 25 µg (SXT), Tetracycline - 30 µg (TE), Teicoplanin - 30 µg (TEC) and Vancomycin - 10 µg (VAN). International reference strains were used with a positive control (*E. faecium* BM 4147 vanA genotype) and a negative control (*S. aureus* ATCC 25923). The zone of inhibition was interpreted according to CLSI (2014), and ascribing the isolates the categories susceptible (S), intermediate (I) and resistant (R).

MIC test of milk samples

The intermediate enterococci samples to VAN were tested with the minimum inhibitory concentration (MIC) method, which exhibits a higher accuracy than the disk diffusion method (Bauer et al., 1966). The commercial kit E-test™ was used in Mueller-Hinton agar, following the manufacturer's recommendations (Bauer et al., 1966; CLSI, 2014).

Multiresistance classification

The enterococci isolates that were simultaneously resistant to at least three different groups/classes of antimicrobials were designated multi-resistant.

Statistics

Descriptive statistics were used through the distribution of absolute frequencies relative to the results obtained in the *in vitro* microbial sensitivity tests. Fisher´s exact test was performed to verify if there were differences between the percentage of multiresistant strains from bulk tanks and mastitis samples. Kruskal Wallis test was used to verify if the median number of antimicrobial compounds to which the isolates were resistant differed between bulk or mastitis isolates. The significance level of 0.05 was considered for the analyses.

RESULTS AND DISCUSSION

None of the isolates were resistant to VAN, while 53 isolates were intermediate resistant (all bulk). These 53 intermediate resistant enterococci showed no resistance by the minimum inhibitory concentration (MIC) method.

VRE are frequently isolated in hospital environments, and the studies related to *in vitro* antimicrobial sensitivity of enterococci isolated from bovine clinical mastitis have shown a low prevalence of resistant to VAN (Różańska et al., 2019), corroborating our findings. In Turkey, enterococci isolated from clinical mastitis showed 1.1% of isolates resistant to VAN (Erbas et al., 2016). In Poland, 426 enterococci from clinical mastitis showed 0.94% of resistance to this drug (Różańska et al., 2019). However, a significant prevalence was observed in Slovakia, with 15.2% of enterococci from bulk tanks resistant to VAN (Fabianová et al., 2010). The same study also found a high prevalence of enterococci susceptible to TEC, as was detected in isolates from bulk tanks (99.6%) and in all isolates from mastitis (Table 1).

Regarding the multiresistant strains, 245 isolates were isolated from bulk tanks (95%) and 47 from clinical mastitis (90.4%). Of these, 74.6% (bulk tanks) and 72.3% (mastitis) were resistant for at least one drug among five or more classes of antibiotics tested (Table 2).

The isolates showed high percentages of resistance to the antibiotics most commonly used on farms for the treatment of animals: ceftiofur (88.5%), tetracycline (57.7%) and neomycin (48.1%). This result is an indication of the difficulty in treating clinical mastitis on farms and the recurrence of cases annually.

In Table 1, the multiresistance profile of bulk tanks isolates are shown. More than 90% of the isolates were resistant to CL (93.8%), NOV (98.8%), CFO (91.9%), and OXA (91.9%). Among isolates from bovine mastitis, the highest resistance was identified to SXT (84.7%), NOV (100%), CFO (88.5%), and OXA (80.8%).

Drugs like PEN, TE, Cephalosporins (CL and CFO),

Table 1. *In vitro* antimicrobial sensitivity profile of *Enterococcus* spp. isolated from bovine clinical mastitis (52 samples) and from bulk tanks milk (258 samples) in dairy farms from São Paulo and Minas Gerais states, Brazil, in 2018 and 2019.

Classes	Antimicrobian	Profile sensitivity					
		Bulk tanks			Clinical mastitis		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Aminocoumarins	Novobiocin	1 (0.4)	2 (0.8)	255 (98.8)	0 (0)	0 (0)	52 (100)
Aminoglycosides	Gentamicin	106 (41.1)	75 (29.1)	77 (29.8)	29 (55.8)	1 (1.9)	22 (42.3)
	Neomycin	24 (9.3)	58 (22.5)	176 (68.2)	26 (50.0)	1 (1.9)	25 (48.1)
Cephalosporins	Cephalexin	6 (2.3)	10 (3.9)	242 (93.8)	41 (78.8)	3 (5.8)	8 (15.4)
	Cefoxitin	12 (4.6)	9 (3.5)	237 (91.9)	5 (9.6)	1 (1.9)	46 (88.5)
Fluoroquinolones	Ciprofloxacin	102 (39.5)	141 (54.7)	15 (5.8)	44 (84.6)	2 (3.9)	6 (11.5)
	Enrofloxacin	34 (13.2)	199 (77.1)	25 (9.7)	41 (78.9)	9(17.3)	2 (3.8)
	Marbofloxacin	75 (29.1)	171 (66.3)	12 (4.6)	28 (53.8)	7(13.5)	17 (32.7)
Glycopeptides	Vancomycin	205 (79.9)	53 (20.1)	0 (0)	52 (100)	0 (0)	0 (0)
	Teicoplanin	257 (99.6)	0 (0)	1 (0.4)	52 (100)	0 (0)	0 (0)
β-Lactams	Ampicillin	256 (99.2)	0 (0)	2 (0.8)	52 (100)	0 (0)	0 (0)
	Penicillin	252 (97.7)	0 (0)	6 (2.3)	45 (86.5)	0 (0)	7 (13.5)
	Oxacillin	7 (2.7)	14 (5.4)	237 (91.9)	5 (9.6)	5 (9.6)	42 (80.8)
Sulfonamides	Sulfametoxazole/Trimetoprim	88 (34.1)	4 (1.5)	166 (64.3)	6 (11.5)	2 (3.8)	44 (84.7)
Tetracyclines	Tetracycline	118 (45.7)	0 (0)	140 (54.3)	21 (40.4)	1 (1.9)	30 (57.7)

S, Susceptible; I, intermediate; R, resistant; %, percentage.
Source: Różańska et al., 2019

Table 2. Classification of multiresistant *Enterococcus* spp. isolated from bovine clinical mastitis and bulk tanks resistant to three, four, five, six or seven different classes of antibiotic, in dairy farms from São Paulo and Minas Gerais states, Brazil, in 2018 and 2019.

The quantity of antibiotic classes	Enterococci - n (%)	
	Bulk tanks	Clinical mastitis
3	10 (4.1)	2 (4.3)
4	52 (21.2)	11 (23.4)
5	99 (40.4)	12 (25.5)
6	76 (31.0)	10 (21.3)
7	8 (3.3)	12 (25.5)
Total	245 (100)	47 (100)

Source: Różańska et al., 2019

and Fluoroquinolones (ENR, MRB and CIP) are frequently used in dairy farms, therefore, study of the susceptible profile of these antibiotics is very important (Beuron et al., 2014; Stevens et al., 2016). A low prevalence of resistance to PEN was identified, which corresponded to 13% of isolates from clinical mastitis and 2% of samples from bulk tanks. Regarding TE, 54% of isolates from bulk tanks and 57% from clinical mastitis were resistant. Różańska et al. (2019) also showed a low percentage of resistant strains from clinical mastitis to PEN (2.58%). The high prevalence of resistant strains to cephalosporins in this study should be noted (Table 1).

Resistance to fluorquinolones was low in strains from bulk tanks and clinical mastitis (less than 10% of isolates). However, there was a high percentage of intermediate resistance in the isolates from bulk tanks, 54.7, 77.1, and 66.3%, respectively to CIP, ENR, and MRB. Of the clinical mastitis isolates, 11.5 and 32.7% were resistant to CIP and MRB, respectively (Table 1). And for CIP, Różańska et al. (2019) noticed 0.47% of enterococci resistant from clinical mastitis.

Fabianová et al. (2010) reported 8.7 and 4.7% of resistant enterococci from bulk tanks to GEN and AMP, respectively. In this study, a low percentage of resistant strains were observed to AMP, only 0.8%. For GEN, 29.8% were resistant, while 29% presented an intermediate resistance (Table 1).

Erbas et al. (2016) noticed a high prevalence of resistant enterococci to TE, 81% of isolates isolated from clinical mastitis, while Różańska et al. (2019) observed 61.5%. In this study 57.7% of resistant strains were identified as shown in Table 1.

According to Fisher's test, the percentage of multidrug-resistant strains in the tanks was 94.96% and in the animals was 90.38%. There was no statistical difference, with a p value of 0.1986. According to the Kruskal Wallis test, the median number of resistant compounds of the tank was equal to 6, and the median number of resistant compounds of the animals with mastitis was 5. There was no statistical difference in the median of the two groups, and the p value was 0.1350. In summary, there was no considerable statistical difference between isolates originating from bulk tanks and from animals with mastitis. We suggest that milk samples collected from the tanks may be adequate for antimicrobial sensitivity profiling research, as they are simpler and less expensive for farmers.

Data about antimicrobial sensitivity of *Enterococcus* spp. from milk from cattle, in bulk tanks or clinical mastitis, are scarce. Studies related to this topic indicate that there are many factors involved in the emergence of resistant strains, and that this will depend on the farm, the management performed, and the antimicrobial treatment applied (Różańska et al., 2019; Erbas et al., 2016; Fabianová et al., 2010).

In Brazil, about 20 to 30% of milk and dairy products are sold without any heat treatment (IBGE, 2015). Our results show that more than 90% of enterococci from bulk tanks, which are milk ready for use are multiresistant, indicating risks to public health through ingestion of contaminated milk. In addition, the presence of these enterococci infecting cows could lead to horizontal transfer of resistance genes to other Gram-positive bacteria present in the mammary glands.

The treatment and the preventive use of antimicrobials in bovine mastitis cases are associated with the indiscriminate use of these drugs, and probably this is the main reason for the increase of resistant strains in cattle farms (Krömker and Leimbach, 2017). Many studies connect the indiscriminate use of antibiotics in animal production with the dissemination of multiresistant *Enterococcus* spp. Therefore, the transmission of these pathogens by foods is a big risk to public health (Tavaras et al., 2012; Ali et al., 2013; Różańska et al., 2019).

More studies are needed to identify the risk factors associated to multiresistant *Enterococci* spp. causing bovine mastitis, including the use of genetic analyses able to characterize the enterococci species. This study might assist future researches on the impact of enterococci in animal and public health, as well as guide which treatment protocols can be used in cases of bovine mastitis.

Conclusion

No *Enterococcus* spp. resistant to vancomycin was detected. However, a high multiresistant enterococci strains were detected in bulk tanks (95%) and from clinical mastitis cases (90.4%), pointing to the risks of misuse of antibiotics in dairy cattle farming. These data highlight the need for more studies on the impacts of enterococci infections in both human and animal health, especially towards the possibility of transmission of resistance genes from *Enterococcus* spp. to other pathogenic bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

COVID-19 and pregnancy: Investigation of serological markers and associated factors

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COVID-19 is a major public health emergency that has recently shaken the world. Pregnant women have not been spared from this pandemic. Although there is no clinical difference between them and the general population, pregnant women are at increased risk of developing severe forms and pregnancy-related complications. The objective of this study was to investigate the presence of anti-SARS-CoV-2 antibodies and to identify associated factors in pregnant women followed at the Regional Hospital Center (CHR) of Saint-Louis, Senegal. A cross-sectional, prospective and descriptive study was conducted among 400 pregnant women followed at the Saint-Louis Regional Hospital (Senegal) over a five-month period from March to July, 2021. None of the patients had received an anti-COVID-19 vaccine. Determination of anti-SARS-CoV-2 antibodies was performed by the *Healgen IgG/IgM SARS-CoV-2* qualitative rapid test. 400 pregnant women were included in the study. The mean age was 27.8 years (±6.3). The most representative age group was between 25 and 29 years with 29%. The majority of patients (76.8%) were housewives. Only 41 patients (10.3%) had travelled outside Saint-Louis (Senegal) in the previous 6 months. None of the patients had received anti-COVID-19 vaccine. Anti-SARS-CoV-2 antibody testing was positive in 232 cases (58%). IgG anti-SARS-CoV-2 antibodies were present in all the 232 women (100%) and IgM was in 6 cases (2.6%). The study showed a high seroprevalence of anti-SARS-CoV-2 in pregnant women followed at the Saint-Louis Regional Hospital (Senegal) showing a large underestimation of the pandemic in that population. Further evaluation on the role of SRAS-CoV-2 antibodies in the protection against the virus or outcome of pregnancy women need to be investigated on a larger size of pregnant women.

Key words: Seroprevalence, SARS-CoV-2, pregnant women, Saint-Louis, Senegal.

INTRODUCTION

SARS-CoV-2 infection is a global health emergency. First appearing in China's Hubei province in late 2019, it has spread rapidly around the world. As of November 2022, the World Health Organization (WHO) has recorded 636 million infected people worldwide with 6.6 million deaths (Statistics of Coronavirus in the World, 2022). On the

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same time, Senegal recorded 88859 infected people including 86858 recovery persons and 1968 deaths (Pandémie COVID-19/Sénégal: Communiqué994, 2022). SARS-CoV-2 infection is responsible for variable clinical pictures that can range from simple forms associating fever, cough, myalgia, headache and possibly digestive disorders as well as severe forms responsible for an acute respiratory distress syndrome that can lead to death (Peyronnet et al., 2020). Several risk factors for the development of acute respiratory distress syndrome, such as advanced age, male gender, presence of comorbidities, oxygen desaturation, and abnormal biological parameters (elevated LDH, elevated procalcitonin, low CD4 count, hypoalbuminemia) have been identified in the literature (Chen et al., 2020a). However, data on COVID-19 in pregnant women remain limited (Flannery et al., 2020).

Early study in China series did not appear to show any difference in COVID-19 positive in pregnant women compared to the general population clinically. However, it appears that SARS-C-oV-2 positive pregnant women are at increased risk of developing severe forms and pregnancy-related complications (Moore and Suthar, 2021; Peyronnet et al., 2020). Intrauterine maternal-fetal transmission has not been reported but cases of early infected neonates suggest probable vertical transmission per-partum or neonatally (Peyronnet et al., 2020).

The objective of this study was to investigate the presence of anti-SARS-CoV-2 antibodies and to identify COVID-19 associated factors in pregnant women followed at the Regional Hospital Center of Saint-Louis (Senegal).

METHODOLOGY

The study population is selected with ancillary study of the seroepidemiological survey of hepatitis E conducted among pregnant women in Senegal. The enrollment of participants was carried out from March to July 2021 at the regional hospital of Saint-Louis (Senegal). Socio-demographic characteristics and inclusion criteria of the participants were fully described (Viruses 2022, 14, 1742. https://doi.org/10.3390/v14081742) (Diouara et al., 2022). The free and informed consent of the pregnant women was obtained verbally, individually. The management of the information was done in strict compliance with medical secret.

Ethical and administrative authorization was obtained from the National Health Research Ethics Committee of Senegal (N°000130/MSA/CNRES/Sec).

For sample collection and processing, 400 pregnant women were recruited in this study. Determination of anti-SARS-CoV-2 antibodies (in the residual plasma samples collected as part of the hepatitis E seroepidemiological study) was performed by the *Healgen IgG/IgM SARS-CoV-2* rapid qualitative test according to the manufacturer's instructions. This assay uses lateral flow technology for the qualitative and differential detection of anti-SARS-CoV-2 IgM and IgG antibodies. All plasma samples were biobanking in -80 until their use. From the data collected, the following parameters were investigated:

1. Socio-demographic aspects: Age, marital status and regime, profession, level of education, socio-economic level, travel outside Saint-Louis (Senegal) in the last 6 months, gestational age, anti-COVID-19 vaccination.
2. Serological aspects: Presence or absence of anti-SARS-CoV-2 IgM and/or IgG.

Data entry and analysis were done using Excel and Epi info7 software. Categorical variables were expressed as proportions and numerical variables as median and standard deviation.

RESULTS

400 pregnant women were included in the study. The mean age was 27.8 years (±6.3). The most representative age group was between 25 and 29 years with 116 cases (29%) (Figure 1). They were married in 392 cases (98%) under monogamous regime with 317 cases (81%). The majority of patients were housewives with 307 cases (76.8%). Only 41 patients (10.3%) had travelled outside Saint-Louis (Senegal) in the last 6 months. No patient had received a COVID-19 vaccine. The median gestation was 2 with extremes of 1 and 11. Primiparous women were in the majority with 115 cases (28.8%) (Table 1).

Concerning seroprevalence of SRAS-CoV-2 in pregnancy women, all patients were tested for the presence of anti-SARS-CoV-2. They were positive in 232 cases (58%) and IgG anti-SARS-CoV-2 antibodies were present in all of those 232 cases (100%). However, 6 (2.6%) of them had simultaneous IgM anti-SARS-CoV-2 antibodies.

DISCUSSION

The COVID-19 pandemic has resulted in a global health crisis that has impacted all areas of life. Its rapid progression and the lack of knowledge of its impact on pregnancy at the beginning of the pandemic led obstetricians to adapt their practice. Current knowledge suggests that there is no risk of maternal-fetal transmission of SARS-CoV-2 (Chen et al., 2020b). However, cases of COVID-19-infected newborns diagnosed from samples taken several hours after birth have been published. Some studies point to the possibility of vertical transmission through the detection of IgM in the serum of newborns of infected mothers (Zeng et al., 2020).

However, samples of the amniotic fluid and cord blood of the newborn show the absence of the virus in the majority of cases of infection that occur during the third trimester of pregnancy. Information on patients exposed in early pregnancy is currently scarce (Dong et al., 2020). Teratogenicity of SARS-CoV-2 appears to be unlikely but may increase the risk of growth retardation and fetal death in utero. Hence the need for strict monitoring of the fetal growth curve, particularly during the third trimester of pregnancy (Schwartz and Graham, 2020). The clinical course of SARS-CoV-2 infection in pregnant women is similar to that of non-pregnant women, although fever

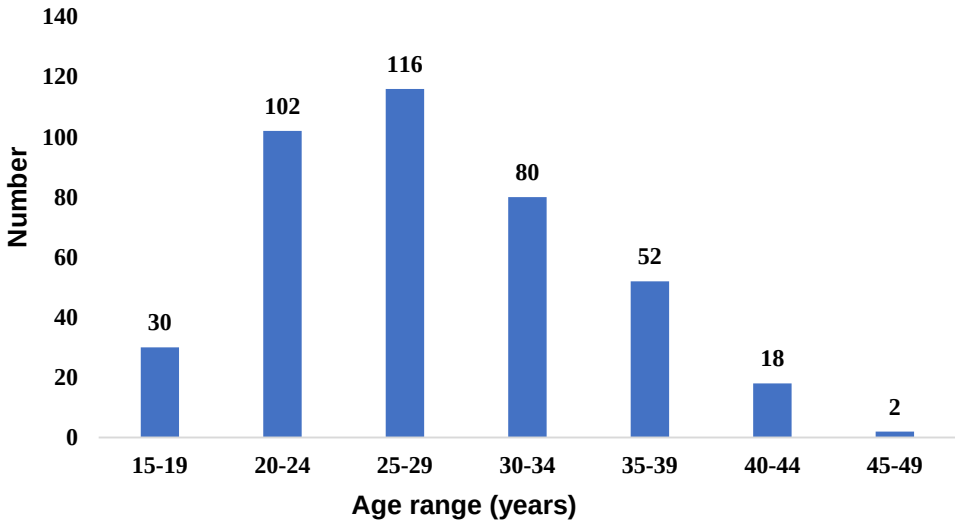

Figure 1. Distribution of patients by age range.
Source: Authors

Table 1. Distribution of patients according to socio-demographic data.

Socio-demographic data	Number	Percentage (%)
Marital status (n=400)		
Unmarried	8	2
Married	392	98
Monogamous	317	81
Polygamous	74	19
Travelled in last 6 months (n=400)		
Yes	41	10.3
No	359	89.7
Number of pregnancies (n=400)		
1	115	28.8
2	96	24
3	76	19
4	58	14.5
5	27	6.7
Others	28	7

Source: Authors

appears to be less prevalent than in the general population (Kouas et al., 2020).

During pregnancy, physiological and immunological changes make pregnant women more susceptible to viral respiratory infections and severe pneumonia. Studies done during the SARS-CoV-1 and MERS-CoV epidemics showed that pregnant women were at higher risk of developing severe complications (Juan et al., 2020; Lambelet et al., 2020).

In our series, the mean age of the patients was 27.8 years (±6.3). In Ethiopia, Assefa et al. (2021) found a mean age of 23.9 years. The majority of our patients were housewives (76.8%), as in a Cameroonian study where 44% of pregnant women were unemployed (Moustapha et al., 2022). Precariousness and promiscuity are risk factors for the occurrence of SARS-CoV-2 infection in pregnant women (Ngaba et al., 2021). For diagnosis of SARS-CoV-2 infection, PCR (direct diagnosis) and serological tests (indirect diagnosis) are used (Hantz, 2020). A disadvantage of indirect diagnosis

is the limited sensitivity at an early stage, when the host has not yet developed specific antibodies. Indeed, during SARS-CoV-2 infection, data from the literature have shown antibody production beginning after the first week of infection and generally detectable from the second (Van Elslande et al., 2021). IgM type antibodies appear from the 7th day and IgG type antibodies from the 10th day (Hantz, 2020). IgG will persist in circulation for months (Sh. Nur et al., 2022). In this study, the seroprevalence of anti-SARS-CoV-2 was 58%. This seroprevalence was much higher than those found in the literature in Philadelphia and Ethiopia with respectively 6.2 and 5.7% (Assefa et al., 2021; Flannery et al., 2020). In Somalia, seroprevalence of 36.7% was found by Sh Nur and al. (2022). In a Parisian hospital, four months after the beginning of the epidemic, the seroprevalence of IgG was 4.7% among women giving birth (Tsatsaris et al., 2021).

The results provide sufficient evidence that the pregnant women followed at the hospital were largely affected by COVID-19. They had not developed severe forms that could require hospitalization. In Senegal, the same observation was made by Diouf et al. (2020) who did not find any severe forms in their study.

Indeed, in Jering's study, about 5 to 10% of pregnant women had a severe form of the disease, 4% were admitted to intensive care and 3% required mechanical ventilation (Jering et al., 2021). They also had more heart attacks and venous thromboembolic events than pregnant women not infected with SARS-CoV-2 (Jering et al., 2021). Certain comorbidities such as advanced maternal age, high body mass index, pre-existing hypertension and diabetes mellitus are risk factors for the occurrence of severe forms of COVID-19 in pregnant women (Jering et al., 2021). Indeed, the risk of infection and development of other complications is high during pregnancy. Despite the possibility of passive immunization, the best prevention to protect the mother from SARS-CoV-2 infection is vaccination, which will provide the fetus and the newborn with strong and effective protection against infection through passive placental transfer of specific antibodies (Zambrano et al., 2021). Placenta functions as an immunological barrier preventing viral transfer to the fetus, but allows the transfer of immunological components, such us immunoglobulins. In addition, the rate of seroprevalence in the newborns demonstrates an active communication between maternal immune system and fetus (Zambrano et al., 2021).

Conclusion

The study showed a high seroprevalence of anti-SARS-CoV-2 antibodies in pregnant women followed in Saint-Louis (Senegal) showing a large underestimation of the pandemic in that population. Cross-tabulations between different parameters reveal no associated factors with such high prevalence. Further evaluation on the role of SRAS-CoV-2 antibodies in the protection against the virus or outcome of pregnancy women need to be investigated on a larger size of pregnant women.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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African Journal of Microbiology Research

Full Length Research Paper

The role of effector Ssc1 in *Sclerotinia Sclerotiorum* and pathogenicity of *Botrytis cinerea* in the early infection stages

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In this experiment, the action mechanism of the gene *Ssc*1 was studied in the process of interaction with plants through heterologous expression, subcellular localization and fluorescent PCR technology. It was found that the gene *Ssc*1 could enhance the pathogenicity of *Botrytis cinerea* through heterologous expression. Fusing the promoter, SP and CTP of *Ssc*1 with GFP and expressing in tobacco, it was found that the fusion protein could be secreted into plant cells and located in chloroplasts. Trypan blue staining and fluorescence detection found that in the early stage after inoculation and in the areas outside of the scab, GFP fluorescence could be detected in the tobacco leaves despite the trypan blue staining being negative. Additionally, it was proved by quantitative PCR that the gene *Ssc*1 was highly expressed in the early infection stages. Taken together, these results indicated that the effector *Ssc*1 was an important pathogenic factor, which could locate in chloroplasts and mainly play the role in the early stage during the interaction between *S. sclerotiorum* and plants.

Key words: *Sclerotinia sclerotiorum*; effector; chorismate mutase; heterologous expression; subcellular localization.

INTRODUCTION

Sclerotinia sclerotiorum (Lib. de Bary) is an important necrotrophic phytopathogenic fungus with a wide host range of more than 400 plants, including rape, sunflower, legumes, *Cucurbitaceae* and *Solanaceae* (Boland and Hall, 1994; Derbyshire, 2022), with infection mechanism similar to that of *Botrytis cinerea* (Pers.) (Amselem et al., 2011). *S. sclerotiorum* is intensely destructive to its host and oxalic acid and cell wall-degrading enzymes play important roles in its infection process (Bateman and Beer, 1965). In addition to cell wall-degrading enzymes, oxalic acid and other traditional pathogenic factors, researchers have found that secretory effector play an

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important role in the interaction of *S. sclerotiorum* with plants (Hancock, 1966; Riou et al., 1991; Kim et al., 2011). For example, Guyon et al. (2014) identified 78 potential effector candidates by secretome analysis andanalyzed the expression patterns of 16 secretory proteins in different plants, Lyu et al. (2016) demonstrated that a small molecular effector protein, SsSSVP1, could be beneficial to self-infection by affecting plant energy metabolism. Wang Xin-yu et al. (2009) found that the effector protein *Sspg1* could interact with IPG-1, a small protein in plants, playing an important role in early pathogenic stage. Zhu et al. (2013) found that *SSITL*, a secretory effector protein, could inhibit the disease resistance mediated by jasmonic acid in the early stages of infection. Fan et al. (2021) Found that a novel effector protein SsERP1 inhibits plant ethylene signaling to promote *Sclerotinia sclerotiorum* infection.

Chorismate mutase catalyzes the conversion of chorismate to prephenic acid, providing precursors for the synthesis of phenylalanine and tyrosine (Andrew, 2003). Although chorismate mutase is ubiquitous in microorganisms and plants and is required for the synthesis of some essential amino acids, not all plant pathogenic microorganisms have this enzyme (Romero et al., 1995). Moreover, the *Cmu1* gene, which is homologous to chorismate mutase, has been shown to be closely related to pathogenicity in the biotrophic pathogenic fungi *Ustilago maydis*. *Cmu1* was also found to influence the salicylic acid (SA) level and then weaken the disease resistance signal (Armin et al., 2011). A novel chorismate mutase from *Erysiphe quercicola* performs dual functions of synthesizing amino acids and inhibiting plant salicylic acid synthesis (He et al., 2021). For nematodes, in addition to providing nutritional needs, CM also provides some help for its parasitic life (Lander et al., 2020).

Previous studies (Dickman Marty Lab.) discovered that the effector *Ssc1*, which is homologous to chorismate mutase, is related to the pathogenicity of *S. sclerotiorum* through deletion mutation (unpublished), but the mechanism is not clear.

Thus, in order to further explore the action mechanism of the gene *Ssc*1, the secretory, subcellular localization, and specific expression at different infection stages were analyzed in this experiments. The results showed that the gene *Ssc*1 could enhance the pathogenicity of *B. cinerea* through heterologous expression. Also it was found that *Ssc*1 proteins could be secreted into the host cells and colocalized with the chloroplast. Finally, through GFP fluorescence detection, trypan blue staining and quantitative PCR, it was proved that the gene *Ssc*1 were highly expressed in the early infection stages. All these indicated that the gene *Ssc*1 was an important pathogenicity factor and mainly play the role in the early stage during the interaction between *S. sclerotiorum* and plants.

MATERIALS AND METHODS

Materials and primers

S. sclerotiorum 1980, *B. cinerea* T_4 and the vector pCX62, pBluntNAT-GFP1-1 were obtained from Prof. Dickman Marty of Texas A&M University. The *Escherichia coli* derivative DH5α was used for cloning purposes. *S. sclerotiorum* 1980 and *B. cinerea* T_4 were routinely cultured on potato dextrose agar (PDA) at 25°C. All chemicals used were of analytical grade. All the primers used in this experiment are listed in Table 1.

Molecular techniques and sequence analysis of the *Ssc*1 gene

Fungal RNA and DNA were extracted using the TRIzol or cetyl trimethylammonium bromide (CTAB) protocol. The plasmid DNA was isolated using the plasmid kit (OMIGA) according to the protocol. Phylogenetic tree generation, and DNA and protein sequence alignment and analysis were conducted using DNAman7.0 software (Lynnon Biosoft). Primers were designed using Primer Premier 5.0 (Premier). Fungi were transformed using the method of restriction enzyme-mediated integration (REMI) based on the same method described previously (Zhao et al., 2010; 2011).

Cloning of the *Ssc*1 gene and heterologous expression in *B. cinerea*

The DNA fragment of the *Ssc*1 gene and its promoter was cloned using the primers SCP1 and SCP2, after which the expression vector pCX62-*Ssc*1 was constructed by inserting the fragment into pCX62. They were then digested by *Xho* I and *Hin*d III, respectively, and then transformed into the protoplast of *B. cinerea* T_4 strains as mentioned above (Zhao et al., 2010; 2011). Transformants were selected on PDA plates using hygromycin B at 250 ug/mL, and then the integration of gene *Ssc*1 into the genome of *B. cinerea* and its normal expression were confirmed by PCR and RT-qPCR, respectively. Finally, the transformants were inoculated onto tobacco using the methods of *in vitro* leaf inoculation with mycelial wafer and spraying with conidial suspension as described previously (Zhao et al., 2018; Xu et al., 2011). The culture dishes and nutrient plates were placed in a 25°C, 85% relative humidity, 14 h light /10 h dark cycle phytotron. The lesions were studied from 12 h, and the disease severity was determined by calculating the disease index. The disease grades were as follows: grade 0: no symptoms; grade 1: small infection spots identified on 1–2 leaves; grade 2: small infection spots identified on 3–5 leaves; grade 3: 1–2 leaves began rotting; grade 4: 3–4 leaves began to rot; grade 5: the whole plant has begun to rot.

Cloning and confirming the promoter of *Ssc*1

The promoter element and the *cis* element were analyzed using the online software Promoter 5.0. The DNA sequence of the promoter was cloned by PCR using the XS1-1 and XS1-2 primers, after which the GFP fusion vector was constructed and transformed in the protoplast of *S. sclerotiorum.* The GFP fluorescence in the hyphae of the transformant was detected by confocal fluorescence microscope.

Analysis of the secretory and subcellular localization of the effector

The signal peptide and localization peptide were predicted using

Table 1. All primers used in this experiment.

Name	Sequence	Location	Using and the size of expected fragment
SCP1	CCGCTCGAGCGCAAGGAGGATCCTAATAG	-733	promoter + *Ssc*1(1216bp)
SCP2	CCCAAGCTTTTAAGAAGAAATCGCCCAAAC	483	promoter + *Ssc*1
XS1-1	GCAAGGAGGATCCTAATAGAATC	-733	Promoter(732bp)
XS1-2	TCCCCCCGGGGTTGGGTGATTGAAG	-1	promoter
XS1-3	TCCCCCCGGGGGAGATGGCGAGAGGAG	60	Promoter+SP(793bp)
XS1-4	TCCCCCCGGGGCATGTTGTTCCATTAGGAAGG	138	promoter+SP+CTP(871bp)
XS1-5	GCATGTTGTTCCATTAGGAAGG	138	mutation screening
HPH1	ATGAAAAAGCCTGAACTC	hph	Transformant screening(1000bp)
HPH2	CTATTCCTTTGCCCTCGG	hph	Transformant screening
Ssc1-1	ATGAAATTCACCACCATTTC	1	*Ssc*1
Ssc1-2	TTAAGAAGAAATCGCCCAAAC	483	*Ssc*1
Ssc1-3	CCCCTCCTACACCCTTCCT	154	Real-time PCR(178bp)
Ssc1-4	GGCACATTCACATCACCCA	332	Real-time PCR
b-t1	TTGGATTTGCTCCTTTGACCAG	b-tubulin	Real-time PCR(104bp)
b-t2	AGCGGCCATCATGTTCTTAGG	b-tubulin	Real-time PCR

Source: Authors

online bioinformatics software, including the SignalP 4.1 Server, PredictNLS and ChloroP 1.1 Server. The fragments containing the promoter+signal peptide (SP) and the promoter+signal peptide (SP) +localization peptide (CTP) were then cloned by PCR using the primer pairs XS1-1/XS1-3 and XS1-1/XS1-4, respectively. Nextly, the GFP fusion vector was constructed and transformed into the protoplast of *S. sclerotiorum* as mentioned above. When inoculating tobacco with the transformant, the fusion protein localization was detected through confocal fluorescence microscope.

Detecting the secretory time and tissue specificity of the *Ssc*1 effector through trypan blue staining

At different times after inoculation with the transformant, in which the GFP fusion protein could be expressed, the leaves of tobacco were dyed using trypan blue and then analyzed by confocal fluorescence microscope.

Detection of transcriptional differences of Ssc1 gene at different stages and locations

Following inoculation on tobacco with the wild type of *S. sclerotiorum*, the mycelia were collected from the areas of the early stage of inoculation site, within the scab and outside the scab, after which RT-PCR of the *Ssc*1 gene was conducted using the primer set *Ssc1*-1/*Ssc1*-2. Additionally, RT-qPCR of the gene *Ssc*1 was performed according to the manufacturer's suggestions, primers were designed according to the sequence of the gene (Table1), and tubulin was used as reference gene for fluorescent qPCR.

RESULTS

The gene *Ssc*1 could heterologously enhance the pathogenicity of *B. cinerea*

A 1216 bp DNA fragment containing the *Ssc*1 gene and its promoter was obtained (Supplementary data 1) by PCR using the primers SCP1 and SCP2 (Table1). The fragment was inserted into the vector pCX62 and transformed into the protoplast of the pathogenic fungus *B. cinerea* T_4 strains. The gene was confirmed to have been integrated into the genome of *B. cinerea* and could be expressed normally by PCR and RT-PCR (Supplementary 2). The pathogenicity of the transformant 3-6-1 was enhanced comparing with that of the wild type following inoculation of tobacco leaves (Figure 1 and Table 2).

The promoter of *Ssc*1 was obtained

The promoter element was analyzed using bioinformatics software, which revealed the presence of a TATA box and a CAAT box in the upstream portion of the gene. The promoter was predicted as shown below.

Promoter predictions for seq 0–2000 bp:
Start End Score
1888 1938 0.98
Promoter Sequence (transcription start shown in bold):
CTCCTTTCTGGTGGCTGTCATATAAGTACGCTCCCAA
CCTCAATGTTCAA

A 733-bp DNA fragment upstream of ATG was cloned by PCR using the XS1-1 and XS1-2 primers (the sequence is shown in Supplementary 2), after which the GFP fusion vector was constructed based on the pBluntNAT-GFP plasmid according to the roadmap (Supplementary 3) and transformed into the protoplast of *S. sclerotiorum*. Some transformants in which the GFP fluorescence was detected through confocal fluorescence microscopy were

Table 2. Disease severity in nutritious pots.

Time	Strains	Total plants	Disease incidence (%)	Disease index	F test	
					0.05	0.01
3dpi	WT(T4)	30	33.3	7.4	ns	ns
	Ssc1+	30	83.3	42.7	**	**
	CK	30	3.3	2.0		
5dpi	WT(T4)	30	50.0	14.7	ns	ns
	Ssc1+	30	100	60.7	**	**
	CK	30	6.7	4.7		

(**: greatly significant difference; *: significant difference; ns: no significant difference. CK, inoculated with water)
Source: Authors

Figure 1. Comparison the pathogenicity of the transformant 3-6-1 (*Ssc*1+) with that of wild-type.
When inoculating on the tobacco leaves, the pathogenicity of the transformate 3-6-1 (*Ssc*1+) was enhanced than that of the wild-type *B. cinerea* T_4 strain. (a):Spraying with conidia suspension; (b): in vitro leaf inoculation with mycelial wafer.
Source: Authors

then obtained (Supplementary 4). Taken together, these findings indicated that the 733 bp DNA fragment upstream of the ATG codon could act as the promoter.

The effector could be secreted and colocalized with the chloroplasts of tobacco

Online bioinformatics software revealed a predicted signal peptide (SP) with 20 amino acids and a chloroplast target peptide (CTP) with 26 amino acids in the gene sequence of *Ssc*1 (Supplementary 5).

Two PCR products that contained promoter+SP and promoter+SP+CTP were cloned by PCR using the primer pairs XS1-1/XS1-3 and XS1-1/XS1-4, respectively. The GFP fusion vector was then constructed according to the roadmap (Supplementary 3), after which it was transformed into the protoplast of *S. sclerotiorum* as mentioned above. A few transformants were obtained, and the recombinant vector was confirmed to have been integrated into the genome by PCR using the primers of HPH1 and HPH2 by PCR in the transformant 3-7-5 and transformant 4-2-8 (Supplementary 6).

Finally, inoculation on the tobacco leaves with transformant 3-7-5, in which GFP was fused with promoter+SP+CTP, revealed that the GFP fusion protein

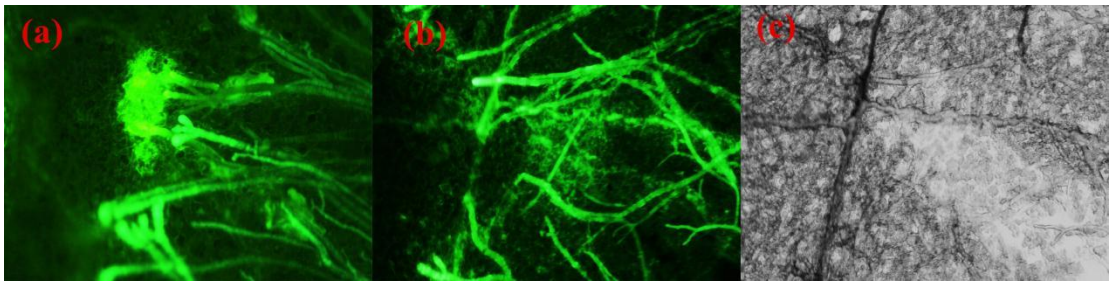

Figure 2. The expression and secretory of the GFP fusion protein in transformant 3-7-5.
When the transformant 3-7-5 in which the GFP fusion vector have been integrated into the genome was inoculated on tobacco, it had been found that the fusion protein could be expressed and secreted into the plant tissue, (a) (b): Fungi hyphae inoculating on tobacco; (c): the result in bright field of vision.
Source: Authors

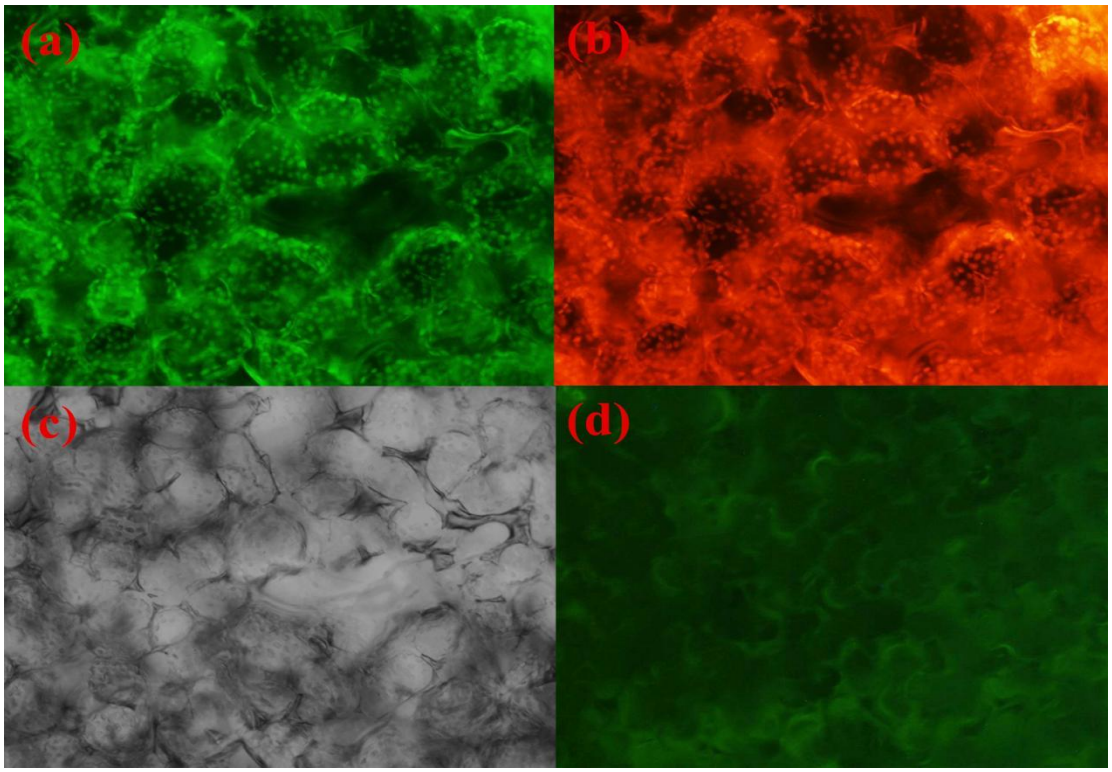

Figure 3. The subcellular localization of the GFP Fusion protein in transformant 3-7-5.
After inoculating the transformant 3-7-5 for 24h, the fluorescence of GFP were colocalized with the Chloroplast. (a): The fluorescence of GFP in the Chloroplast; (b): The autofluorescence of Chloroplast; (c): the result in bright field of vision; (d): The fluorescence of the leaves which not be inoculated with transformant (CK).
Source: Authors

could be expressed, secreted into the host cell, and localized in the chloroplast (Figures 2 to 3). However, the GFP could not be co-localized with the chloroplast when the transformant 4-2-8 was inoculated on tobacco, as the GFP fusion protein had no chloroplast target peptide (Figure 4).

The *Ssc*1 protein were secreted in plant cells in the early infection stage

Following inoculation on tobacco with transformant 3-7-5 for 12 h, GFP fluorescence was detected in the leaves by confocal fluorescence microscopy, despite no scab

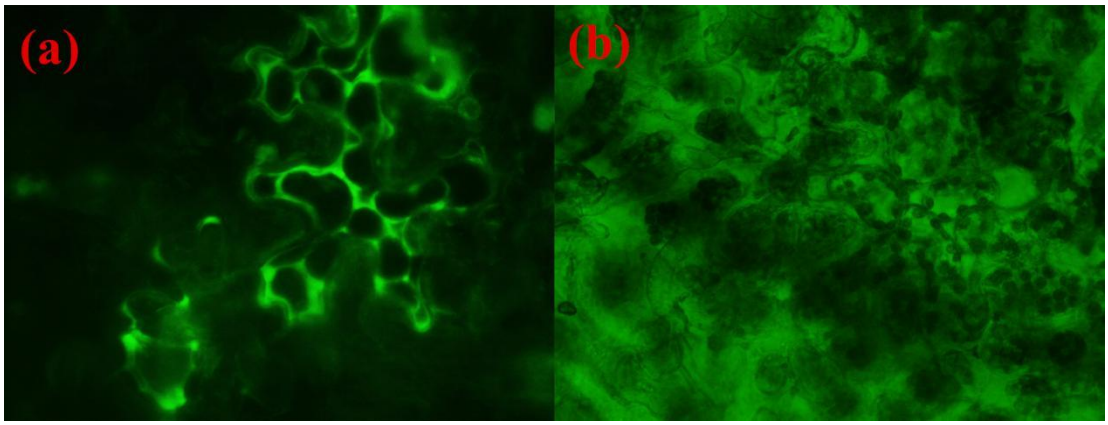

Figure 4. The subcellular localization of the GFP Fusion protein in transformant 4-2-8.
After inoculating the transformant 4-2-8 for 24h, The fluorescence of GFP were not localized in the Chloroplast. (a): The fluorescence of GFP; (b): The autofluorescence of the leaves.
Source: Authors

emerging on the leaf and negative trypan blue staining results (Figure 5). After inoculation for 24 h, a scab began to form, and the leaf tissues of the tobacco and pathogenic hyphae within the scab could be stained by trypan blue, which indicated that the plant cells had died. On the contrary, in the areas outside the scab, although the trypan blue staining of tobacco tissue and the fungal hyphae was negative, GFP fluorescence could be detected (Figure 5). In combination, these findings indicate that although the plant cells were alive in the early infection stage, the fungi had begun to expand; also the effector had begun to be secreted into the plant cells.

The gene *Ssc*1 was highly expressed in the early infection stage

Analysis by RT-PCR and RT-qPCR revealed that the gene *Ssc*1 was highly expressed in the early infection stage and in the areas outside the scab, but its expression was relatively low within the scab, in which plant cells have died (Table 3 and Figure 6).

DISCUSSION

In this study, the gene *Ssc*1 homologous to chorismate mutase in *S. sclerotiorum* was proved to be closely associated with pathogenicity through heterologous expression in *B. cinerea*. The gene encodes a small protein, which could be secreted into plant cells and localized in the chloroplast, playing a role in the early stages of infection.

The shikimate pathway is a fundamental metabolic pathway in plants and microorganisms. The final product of this pathway is chorismate, which is the precursor of many important compounds, including aromatic amino acids (phenylalanine, tryptophan, and tyrosine), salicylic acid (SA), indole-3-acetic acid (IAA) and other secondary metabolites (Strack, 1997). These chorismate-derived compounds (CDCs) play important roles in plant growth, development, defense, and interaction with other organisms (Romero et al., 1995).

Chorismate mutase catalyzes the conversion of chorismate to pre-phenylic acid, providing precursors for the synthesis of phenylalanine and tyrosine (Andrew, 2003). For parasitic pathogenic microorganisms, phenylalanine and tyrosine can be obtained from the host, and thus chorismate mutases may not be necessary for their amino acid metabolism.

However, BLAST searches of the NCBI database revealed that only some pathogenic microorganisms have genes homologous to chorismate mutases, such as nematodes, *U. maydis*, *Erysiphe quercicola*, etc., which have been proved to play a role in the interaction of plant pathogens and plants (Armin et al., 2001; He et al., 2021; Lander et al., 2020).

Armin et al. (2011) found that the *Cmu*1 gene homologous to chorismate mutase was closely related to pathogenicity through affecting the SA level and the resistance signal. It was usually considered that the SA pathway typically plays an important role in the defense against biotrophic pathogens but does not play a major role in resistance to necrotrophic pathogens in plants (Strack, 1997; Govrin and Levine, 2000; Yang et al., 2015). Unlike *U. maydis*, *S. sclerotiorum* is a necrotrophic fungus, as for how this effector play a role in necrotrophic fungus in the process of infection the plant is an interesting question.

Previous studies by the Dickman Lab (unpublished) on the deletion mutation of *Ssc*1 indicated that the *Ssc1* effector was related to pathogenicity. In this study, this

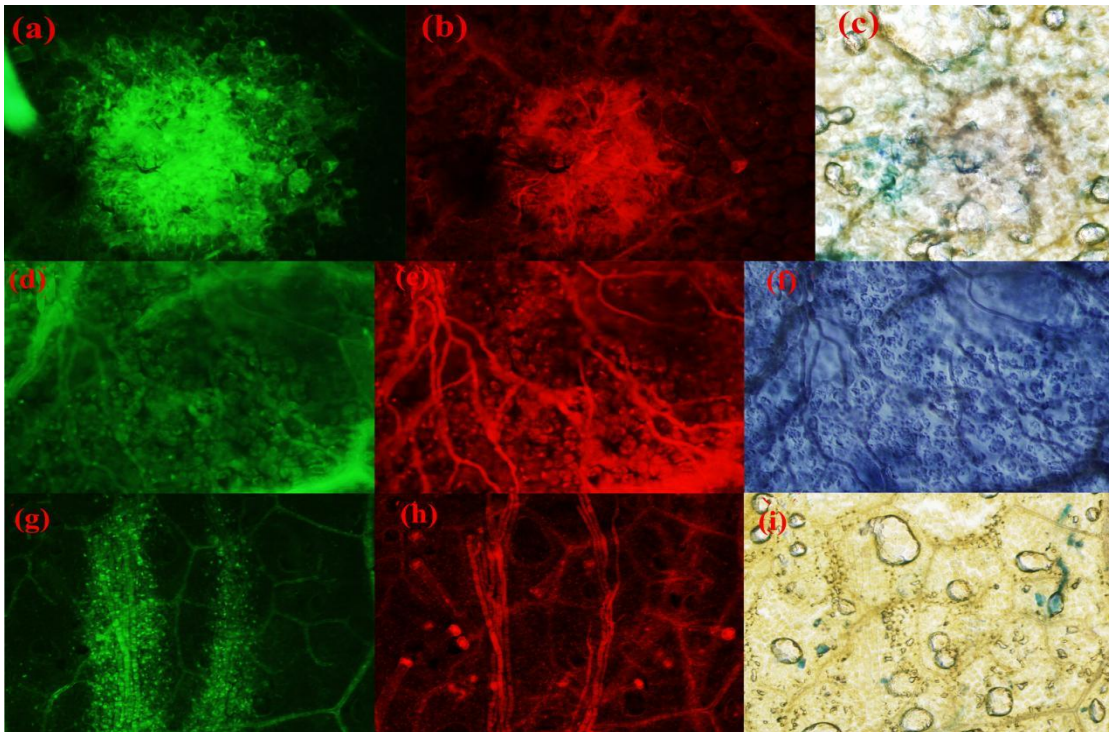

Figure 5. The result of trypan blue staining after inoculating the transformate 3-7-5.
The result of trypan blue staining after inoculating the transformate 3-7-5 for 12h (a): The fluorescence of GFP; (b): The autofluorescence of the plant cells and hyphae; (c): the result of trypan blue staining. The result of trypan blue staining after inoculating the transformate 3-7-5 for 24h(d) (g): The fluorescence of GFP; (e) (h): The autofluorescence of the plant cells and hyphae; (f) (i): The result of trypan blue staining. ((d) (e) (f): within the scab; (g) (h) (i): out of the scab).
Source: Authors

Table 3. The results of RT-qPCR.

Sample	tubulin Ct	choris Ct	ΔCt	ΔΔCt	2-ΔΔCt
Inoculating *S. sclerotiorum* for 2h	28.02	28.41	0.39	0.00	1.00
Outside of the scab	27.65	28.25	0.6	0.29	0.86
Within the scab	27.58	31.61	4.03	3.64	0.08 aA

Lower-case letters stand for the significant difference at the level of 5%; capital letter stand for the highly significant difference at the level of 1%).
Source: Authors

gene was found to enhance the pathogenicity of the pathogen *B. cinerea* through heterologous expression. These findings suggested that the gene is a pathogenic factor rather than a gene necessary for amino acid metabolism. Both *B. cinerea* and *S. sclerotiorum* are necrotrophic pathogens with similar pathogenic mechanisms. Thus, further investigation is needed to determine why only *S. sclerotiorum* retains this important factor related to the interaction between fungi and plants.

In this experiment, within 12 h after inoculation of the transformants 3-7-5 onto tobacco, the mycelia and plant cells could not be stained by trypan blue. Negative trypan blue staining indicated that the plant cells were alive during the early stages of infection. However, GFP fluorescence could be detected in the tobacco leaves despite the trypan blue staining being negative. Through fluorescence detecting, it could be found that the pathogenic hypha had begun to expand near the inoculation point before trypan blue staining positive. It was reported that the necrotrophic pathogens may have a semi-living biotrophic stage, in which stage *S. sclerotiorum* expanded into the plant tissue through its

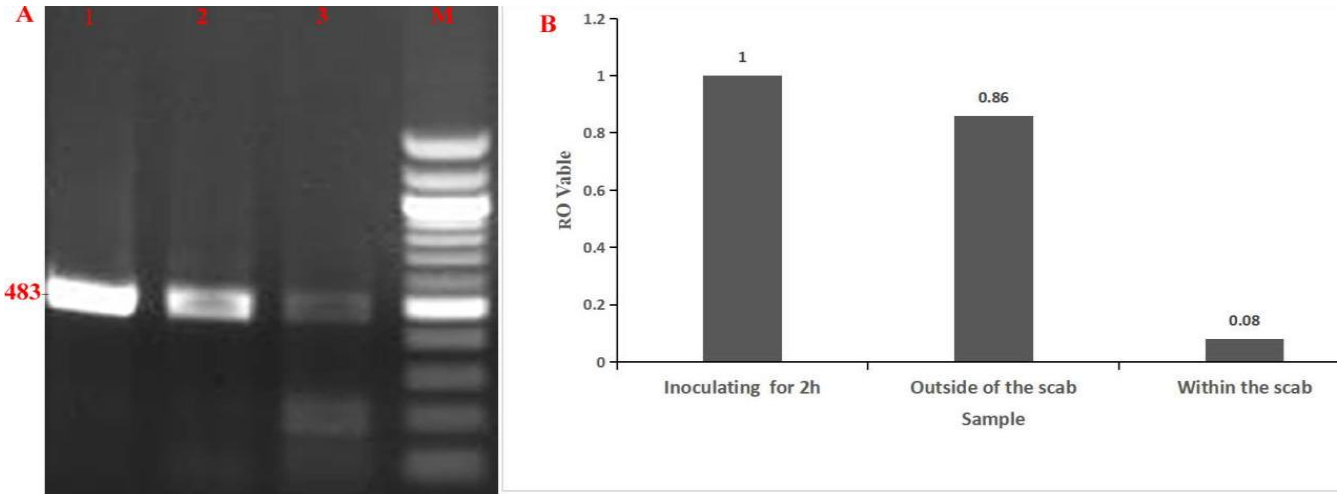

Figure 6. RT-PCR and RT-qPCR of the gene *Ssc*1 in different stage.
(A) The RNA was obtained from the hyphae which 1. when inoculating *S. sclerotiorum* for 12 h; 2 outside of the scab; 3. Within the scab. (B) The RNA used as RT-qPCR were obtained from the leaf tissue which 1. Inoculating *S. sclerotiorum* for 2h; 2. outside of the scab; 3.within the scab.
Source: Authors

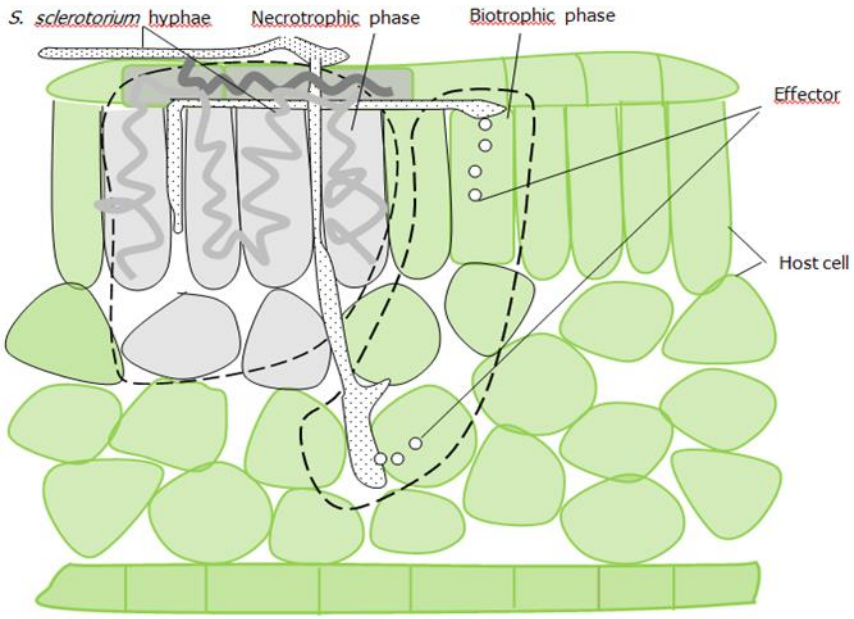

Figure 7. The model of the secretory of *Ssc*1 effector and the infection of the fungus *S. sclerotiorum.*
Source: Authors

adaptation to the plant environment and inhibition of host defense (Mehdi et al., 2015). Effectors produced by the fungus may help its own growth and inhibit host resistance during this semi-living biotrophic stage. It was speculated that the *Ssc*1 effector may influence the SA level and resistance signal by affecting the metabolic pathway of chorismate during this stage, similar to that of the *Cmu*1 gene, and then the mycelium grew and expanded as proposed in Figure 7.

At 24 h after inoculation with the transformant 3-7-5, scabs began to form, and the trypan blue staining of plant cells and pathogenic hypha within the lesion range was

positive, indicating that the cells had died. During this stage, GFP fluorescence was obviously co localized with the chloroplasts of the tobacco, indicating that effector proteins accumulated in chloroplasts (Figure 3). Previous research showed that the response of plants to various stress factors first occurs in the membrane system (Renu et al., 2014). Stress factors, including pathogenic organisms, cause metabolic disorders and accelerate the variation in the biochemical and biophysical structures of membranes, as well as initiate some secondary metabolism related to the stress response (Renu et al., 2014). The chloroplast is an organelle specialized for carrying out photosynthesis in plants, and the Calvin-Benson cycle occurring in the chloroplasts can provide chorismate precursors, such as D-erythritose-4 phosphoric acid and sedum phosphate heptaose (Wang et al., 2006). Perhaps under the pressure of pathogen infection, the chloroplast membrane structure was first disordered, and then the accumulation of some enzymes, including exogenous chorismate mutase, on the imperfect chloroplast affects the secondary metabolism related to plant defense, such as the metabolism related to salicylic acid (SA), IAA and aromatic amino acids (Baier and Dietz, 2005). However, further studies are necessary to determine why the effector protein was located in the chloroplast as well as its role in this organelle.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Dickman M. B. for offering the strains of *S. sclerotiorum* 1980 and *Botrytis cinerea* T4. This work was supported by Nature Science Fund of Shandong Province (ZR2020MC125 , ZR2013CM006); Key R and D plan of Shandong Province (2019GNC106018); and Shandong Provincial Key Laboratory of Agricultural Microbiology Open Fund (SDKL2017015).

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Short legends for supporting information

Supplementary. 1: The DNA sequence of the promoter and gene *Ssc*1
The full length DNA fragment containing the *Ssc*1 gene and its promoter is 1216 bp.
Supplementary. 2 Detecting the integration of the recombinant vector and the expression of *Ssc*1 in the Transformant 3-6-1 by PCR and RT-PCR
(a) : Detecting the integration of the recombinant vector in the transformant 3-6-1 by PCR using primer pairs Ssc1-1/Ssc1-2 (1) vector; (2): Transformant 3-6-1; (3): WT and HPH1/HPH2 (4) vector; (5): Transformant 3-6-1; (6): WT.
(b) : Detecting the expression of *Ssc*1 in the Transformant 3-6-1 by RT- PCR using the primer pair Ssc1-1/Ssc1-2 (1):Transformant 3-6-1 ; (2):wild-type.
Supplementary. 3 Construction of the GFP fusion vector
The fusion vector was constructed basing on pCX62. Firstly, both pCX62 and pBluntNAT-GFP1 were digested with *Bam*H1 and *Xba*1, then linked by T_4 ligase. Secondly, the vector and PCR fragment which containing (1): promoter +signal peptide (SP)+chloroplast localization peptide(CTP); (2):promoter; (3): promoter +signal peptide(SP) were digested with *Bam*H1 and *Xma*1 respectively, and then linked by T_4 ligase.
Supplementary. 4 Detecting the GFP fluorescence in the hyphae of transformant
Detecting the GFP fluorescence in the transformant in which GFP fusion with the nature promoter of *Ssc*1. (a): The fluorescence of the hyphae; (b): The fluorescence of the hyphae in the leaf of tobacco; (c): the result in bright field of vision.
Supplementary. 5 The predicted SP and CTP in *Ssc*1
Online bioinformatics software revealed a predicted signal peptide (SP) with 20 amino acids and a chloroplast target peptide (CTP) with 26 amino acids in the gene sequence of *Ssc*1.
Supplementary.6 Detection of *hph* gene in the transformant 3-7-5 and the transformant 4-2-8 by PCR using the primers pair HPH1/HPH2
M: DNA Mark DL2000; 1: WT; 2: Transformant 3-7-5; 3: Transformant 4-2-8

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Phenotypic characterization and antimicrobial susceptibility testing of *Klebsiella* isolates from *Rattus rattus* captured at university of Abuja metropolis

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The aim of this study is to phenotypically characterize *Klebsiella* isolates from rats. A survey was conducted on 100 swaps samples obtained from captured house rats in University of Abuja main campus from May to August 2021. Isolation and identification of the *Klebsiella* species was done using conventional cultural and biochemical techniques. Of the 100 samples analyzed, 20 (20%) were positive for *Klebsiella*, 12% of the isolates were identified as *Klebsiella Pneumonia* while 8% were identified as *Klebsiella oxytoca*. Isolates were further confirmed by Microbact 24E test kit identification system (Oxiod, UK). *Klebsiella* isolates encountered in this study were further subjected to antimicrobial susceptibility testing using modified single disk diffusion method. Result of the antimicrobial susceptibility testing showed that the *Klebsiella* species were resistant to chloramphenicol (80%), gentamycin (90%), augmentin (80%), Cotrimoxazole (70%) and amoxicillin (70%). However, the Isolates were susceptible to Pefloxacin (100%), Streptomycin (40%), Ofloxacin (100%), Ciprofloxacin (100%) and Sparfloxacin (80%). Conclusively, this study documented the occurrence and existence of multiple resistant strains of *K. Pneumonia* and *K. Oxytoca* in rats in our environment and it is therefore of public health concern.

Key words: *Klebsiella* species, household rat, Rattus *rattus*, antimicrobial susceptibility.

INTRODUCTION

Klebsiella are widely distributed in nature and are part of the normal floral of the gastrointestinal tract of humans and animals (Majumder et al., 2018). They are found in the oropharynx of 1-6% of normal healthy individuals, colonization rate as high as 20% may be seen in hospitalized patients (Arora and Arora, 2012). This colonization may be the source of a good number of lung infections such as severe bronchopneumonia, resulting with chronic destructive and supurative lesions, pleuritis and multiple abscesses in the lungs. High mortality in man and animals is due to septiceamia followed by secondary bacteria invasion (Ernst et al., 2020).

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Klebsiella organisms are often incriminated in most Hospital associated acquired (nosocomial) infections resulting to clinical syndromes such as urinary tract infections, wounds and burns as wounds secondary invaders in other respiratory infections (Majumder et al., 2018). These enteric organisms are also responsible for a significant number of community acquired infections worldwide (Ernst et al., 2020). The organisms are the most frequently encountered gram-negative pathogens causing nosocomial infection of the lower respiratory tract and are second only to *E. coli* as the cause of primary bacteremia associated with gram-negative organisms (Arora and Arora, 2012).

In animals, it is an essential cause of pneumonia, epidemic metritis, cervicitis in mares and septicemia in foals (Wareth and Neubauer, 2021). It has been frequently associated with pneumonia and mastitis in bovine (Piras et al., 2022) leading to high loses in milk production, decreased milk quality and even high mortalities among affected cows (Gorden et al., 2018). Consequently, infection can result in noticeable any monetary involvements in the dairy industry, even in well managed dairy farms (Gorden et al., 2018).

Household rat (*Rattus Rattus*) share living environments with humans and animals, and are predominant in rural areas especially in communities with poor sanitary and environmental conditions (Ogbole et al., 2022). Rodents are infamous for being everywhere since they travel great distances in quest of food between houses and bushes. Domestic rats are omnivores, which makes them susceptible to a number of infectious diseases. Given that rats serve as reservoirs for a variety of bacterial, viral, and parasitic organisms, people are at significant risk of developing virulent diseases linked to *Klebsiella* species (Baidya and Rahman, 2021). Zhong et al. (2020) reported high prevalence of antibiotic resistant *K. pneumonia* from urban rodents and shrews in Southern China. There is paucity of information on the occurrence of *Klebsiella* in rats in the study area. Therefore, this study aimed at the determination of the isolation rate of *Klesiella pneumonia* and *Klebsiella oxytoca* in captured rat in University of Abuja, Federal capital Territory, Nigeria, and the determination of their antimicrobial susceptibilities to commonly used antibiotics.

MATERIALS AND METHODS

Sample collection and preparation

Rat capture

The traditional method for rat captured used was the snap trap method placed in their routes with attractive bait such as roasted fish to attract the rats to the area (Christie et al., 2017). The traps are placed inside closet, under any furniture or bushy passages or other routes that the rats can possibility pass through. This cleverly arrangement lure some of the rats into the traps and have them captured. The captured rats are transferred into a shoebox and transported to the laboratory for further analysis.

Sample collection

The rat was properly restrained by grasping firmly at the base of the tail, by applying tension so that the surface of the rat was grasped using the free hand. The rat was firmly grasped over the shoulder and close to the base of the skull between the thumb and the forefinger (Christie et al., 2017). The vent of the rat was squeezed to remove fecal and urinary materials. A sterilized swap stick was used to aseptically absorbed the material (fecal and urine) and then dipped in peptone water for 24 h for non-selective enrichment. A total of 100 rectal swabs were collected from captured rat using sterile swab sticks in ten different locations within University of Abuja for a period of ten weeks. The rats were captured with local traps. All samples were appropriately labeled, placed in cool thermos and immediately transported to Veterinary Microbiology Laboratory, University of Abuja for laboratory analysis.

Isolation and identification of *Klebsiella*

This study was conducted according to the method adopted by Mailafia et al. (2003). Each swab sticks containing the samples were dipped in test tubes containing 10ml of prepared nutrient broth, and then incubated at 37^0C for 24 h. A loopful of the inoculum was streaked onto already prepared plates of Eosine methylene blue agar (Oxoid, UK) incubated at 37^0C for 24 h. *Klebsiella* species produces large, mucoid, pink to purple colonies with no metallic green sheen on EMB agar. The lactose fermented colonies were picked and sub-culture on nutrient agar plates, incubated at 37^OC for 24 h to obtained pure culture and subsequently inoculated on nutrient agar slant for further characterization.

Presumptive isolates were characterized microscopically using Gram staining and biochemically using catalase, oxidase, triple sugar iron, indole, citrate, methy red, voges proskauer and urea tests as described by Cheesbrough (2006).

Characterization of *Klebsiella* isolates using Microbact 24E Test kits

Using the commercial test kits described by the manufacturers, Microbact™ 24E (Oxoid, UK) isolates were fully biochemically characterized. These biochemical test reactions are included in the Microbact™ identification kits (Oxiod): oxidase, catalase, coagulase, Gram staining, H2S, glucose, mannitol, hylose, indole, urease, VP, citrate, gelatin, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, salicin, arginine and nitrate. The results obtained were interpreted to identify the isolates using the Microbact™ computer aided identification package (Oxiod) supplied along with the kits in combination with the Cowan and Steel's Manual for the Identification of Medical Bacteria (1974). A suspension of the overnight culture of the organism was emulsified in 5ml sterile saline solution and then adjusted to 0.5 McFarland turbidity standards (approximately equal to 1.5×10^8 CFU/ ml of the bacterial suspension).

The wells of the individual substrate were exposed by cutting the end tag of the sealing strip and slowly peeling it backward. The strip was placed in a holding tray, using a sterile Pasteur pipette, 4 drops (approximately 100µm) of the bacterial suspension was inoculated into each well and overlaid with sterile mineral oil. The inoculated rows were sealed with adhesive seal and the specimen identification number was written on the end tag with a marker pen, and then incubated at 37^0C for 18 to 24 h. Following incubation, the reactions were evaluated as positive or negative by comparing it with the color chart. Reactions involving different colours (yellow, red, tan, green, blue) as shown in Figure 1 were observed. The interpretation of the result was based on an octal coding system

Figure 1. Pictorial presentation of Microbact^{TM} identifying the isolates to specie level.
Source: Authors

which was adopted for Microbact. Each group of 3 reactions produces a single digit of the code. Using the result obtained, the indices of the positive reactions were circled. The sum of these indices in each group of the three reactions formed shown against the organism name was the percentage share of the probability for that organism (Sinanjung et al., 2020).

Antibiotics susceptibility test

Antimicrobial susceptibility of *Klebsiella* isolates were tested using the disk diffusion method prescribed by Kirby- Bauer et al. (1966) and in accordance with the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2018). The antibiotics used were cotrimoxazole (30µg), chloramphenicol (30µg), gentamycin (30µg), augmentin (10µg), amoxicillin (30µ), pefloxacin (30µg), streptomycin (30µg), ofloxacin (10µg), ciprofloxacin (30µg) and sparfloxacin (10µg) (Hi media, India). An overnight culture of each isolate was prepared in nutrient broth and incubated at 37 °C for 18 h. The turbidity of the broth was adjusted to McFarland standard of 0.5. The inoculum was then spread on already prepared plates of Mueller Hinton's agar (Oxoid, UK) and left standing for 1-2 min. Using forcep, antibiotics multi-discs (Hi media, India) were aseptically placed on the inoculated plates and then incubated at 37 °C for 24 h. After incubation, the zones of inhibition were measured to the nearest millimeter using a transparent ruler and the values were recorded and interpreted as sensitive, intermediate and resistant according to CLSI, 2018 guidelines.

Data analysis

Statistical Package for Social Sciences (SPSS version 2.6) was used for data analysis. Simple descriptive statistics such as frequency, percentages and tables were used to express the rate of occurrence of *Klebsiella* isolates.

RESULTS

Table 1 shows the biochemical characterization of 20 suspected isolated was carried out as exhibited by *K. pneumonia* and *K. oxytoca* which include Citrate Utilization test Triple Sugar Iron test, Urea test, Methyl Red test, Indole test, and Voges Proskauer test respectively. Table 2 shows that out of the 100 samples analyzed, 20 (20%) yielded growth for *Klebsiella*, 12% of the 20% *Klebsiella* isolates were identified as *K. Pneumonia* while 8% as *K. Oxytoca.* The prevalence and distribution of *Klebsiella* species isolates from rat in

Table 1. Results of Biochemical reactions of 20 suspected isolates of *Klebsiella pneumonia* and *Klebsiella oxytoca* from household Rats (*Rattus Rattus*) in University of Abuja, FCT.

Biochemical test	Number of tested organisms	Number of positive organisms	Number of negative organisms
GR	20	0	20
C	20	20	0
O	20	0	20
TSI	20	20	0
M	20	0	20
U	20	20	0
CI	20	20	0
I	20	8	12
MR	20	0	20
VP	20	20	0

GR – Gram reaction, C- Catalase, O- Oxidase, TSI- Triple Sugar Iron test, M- Motility test, I-Indole test, CI- Citrate test, U- Urease test, MR- Methyl red test, VP- Voges Proskauer
Source: Authors

different location within the University of Abuja Main campus with Faculties of Veterinary medicine and Agriculture showing the highest percentage prevalence while faculties of Health science and Management sciences shows the least percentage prevalence. The prevalence and distribution of confirmed *K. pneumonia* and *K. oxytoca* isolates from rats swab in the University of Abuja Main campus using Microbact 24E identification test kit as seen in Table 3. The microbact test identified the following biochemical test in percentage such as Lysine (100%), Malonate (100%) , Glucose (80%), Xylose (80%), Citrate (80%), and Arginine has the highest percentage that was positive in the five sample tested while Urease (60%), V-P (60%), Gelatin (60%), Lactose (60%), Arabinose (60%) and Raffinose have moderate percentage that was positive. Mannitol (40%), ONPG (40%), TDA (40%), Sorbitol (40%), and Rhamnose (40%) which are slightly moderate in percentage while Ornithine (20%), Indole (20%), and Adonitol (20%) have lower percentage and Salicin (0%) which shows the lowest percentage that was positive of the isolates.

Table 4 shows the antimicrobial susceptibility and resistance of ten antibiotics used in this study. The resistant antibiotics include; Chloramphenicol (CH 30µg), Gentamycin (CN 30µg), Augmentin (AU 10µg), Cotrimoxazole (SXT 30µg) and Amoxicillin (AM 30µg) while the susceptible antibiotics include; Pefloxacin (PEF 30µg), Streptomycin (S 30µg), Ofloxacin (OFX 10µg), Ciprofloxacin (CPX 10µg) and Sparfloxacin (SP 10µg).

Table 5 shows that from the ten antibiotics tested for susceptibility test to the *Klebsiellae* species, it was observed that four isolates dissipated the longest pattern of resistance to seven antibiotics (AM, AU, CH,CN,SP,S and SXT). The next eight isolates dissipated the second longest pattern of resistance to six antibiotics (AM, AU, CH, CN, S, and SXT).

Furthermore, the next two isolates each showed varying resistance patterns namely; AM, AU, CH, CN and SXT displaying five antibiotics, three antibiotics were for AU, CH and CN and one antibiotic was for CN respectively.

DISCUSSIONS

The result of this study conducted at the University of Abuja Campus, Airport road document the occurrence of *K. pneumonia* and *K. oxytoca* from household rats in the school environment. The observed morphological characteristics of the isolates showed typical *Klebsiella* species with circular, dome shaped, mucoid and greyish white colony on nutrient agar. While in EMB agar it gives circular, mucoid and pink purple colonies due glucose fermentation as previously documented by paramedics (2021). The mucoid nature of the colonies is due to the presence of capsular material produced by the organisms (Khaertynov et al., 2018). The overall prevalence of *Klebsiella* species was 20% which indicate the existence of *Klebsiella* species from fecal materials of captured house rats from the University of Abuja campus. This result is closely similar to the study carried out in Ilorin (Fadeyi et al., 2016) and Enugu (Ejikeugwu et al., 2013) all in Nigeria, where they recorded the prevalence of 26.7 and 26.0% respectively. This finding is also lower than the finding of Leangapichart et al. (2021) with a prevalence of 39% from pigs. The distribution of *Klebsiella* organisms in captured house rats shows *K. pneumonia* (12%) to be higher than *K. oxytoca* (8%).These differences could be due to environmental factors that exposes the animal to the organisms and differences in sample size based on the availability of captured house rat in those locations. The presence of *Klebsiella* species should be a source of concern to

Table 2. Prevalence and distribution of *Klebsiella pneumonia* and *Klebsiella oxytoca* by location.

Locations	Number of collected samples	Number of positive organisms	% *K. pneumonia*	Number of positive organisms	% *K. oxytoca*
Faculty of Veterinary medicine	15	2	16.7	1	12.5
Old boys hostel	10	1	8.3	1	12.5
New boys hostel	10	0	0	0	0
Old girls hostel	10	2	16.7	1	12.5
New girls hostel	10	0	0	0	0
Faculty of Agriculture	15	4	33.3	2	0
Faculty of Health Sciences	5	0	0	0	0
Faculty of management sciences	5	0	0	0	0
Faculty of Art	10	2	16.7	2	25
Faculty of Sciences	10	1	8.3	1	12.5
Total	100	12	100(12)	8	100(8)

Source: Authors

public health since rats serve as a reservoir and it accounts for a significant proportion of urinary tract infections, pneumonia, septicemias and soft tissue infections (Massinga et al., 2021).

In this study, the ratio of *K. pneumonia* to *K. oxytoca* was approximately 2:1. This agrees with Fideyi et al. (2016) who had stated that Extended Spectrum Beta Lactam producing *Klebsiella* are mainly caused by the two species *K. pneumonia* and *K. oxytoca* in the ratio of 3:1. This finding may be related to the pathogenicity in association with bacteriophages and hence medical importance of these of *Klebsiella* compared with others (Karumidze et al., 2012).

The prevalence of *Klebsiella* organisms shows Faculty of Agriculture and its environs had highest (33%) while new boys' hostel, new girls' hostel, Faculties of Health and Management sciences had no prevalence (0%). The highest frequency may be due to immunocompromised individual or host and differences in sample size. To a much lesser degree, *K. Oxytoca* has been isolated from rat specimens. Uzoamaka et al. (2017), in Nigeria who confirmed that *K. Pneumoniae* was among the most common causes of lower respiratory tract infections, neonatal septicemias and bacteremia in children. More importantly, *K. pneumonia* is the leading cause of mastitis, metritis and pneumonia in man (Marques et al., 2019). Due to their widespread presence and shared living space with humans and other animals, house rats have been highlighted in this study as important potential carriers of *Klebsiella* infection. Further biochemical test was done to characterize the isolate and differentiate it from other closely related organism and enteric bacteria.

This research also shows that the samples of rectal swab that tested positive for *Klebsiella* species were isolated from immune competent and immune compromised host. This study connotes with the report of Paterson et al. (2004) who stated that *Klebsiella* species are involved in various infections affecting immunocompetent and immunocompromised hosts with a wide spectrum involving urinary, respiratory and gastrointestinal tracts. In addition, they cause bacteremia, septicemia and various organs and soft tissue infections.

During the cause of the study, antimicrobial susceptibility test was performed following the disk diffusion method protocol M27 reference method of National Committee for Clinical Labouratory Standards (NCCLS) with slight modification. It was discovered that *Klebsiella* species are highly resistant to Cotrimoxazole, Chloramphenicol, Gentamycin, Augmentin and Amoxicillin. Massinga et al. (2021) also documented that *Klebsiella pneumonia* is resistant to Amoxicillin-clavulanic acid Gentamicin. They are susceptible to Pefloxacin, Streptomycin, Ofloxacin, Ciprofloxacin and Sparfloxacin. Generally speaking, some of the organisms isolated in this study are resistant to some commonly used antimicrobial drugs. Resistance observed in these studies could be plasmid

Table 3. Identification of the isolate using microbact 24E.

Biochemical test	Number of tested organisms	Number of positive organisms	Number of negative organisms	% of positive organisms	% of negative organisms
Lysine	20	20	0	100	0
Ornithine	20	4	16	20	80
H_2S	20	0	20	0	100
Glucose	20	16	4	80	20
Mannitol	20	14	6	40	60
Xylose	20	16	4	80	20
ONPG	20	6	14	40	60
Indole	20	4	16	20	80
Urease	20	14	6	60	40
V-P	20	14	6	60	40
Citrate	20	18	2	80	20
TDA	20	6	14	40	60
Gelatin	20	14	6	60	40
Malonate	20	20	0	100	0
Inositol	20	4	16	40	60
Sorbitol	20	4	16	40	60
Rhamnose	20	4	16	40	60
Sucrose	20	4	16	40	60
Lactose	20	16	4	60	40
Arabinose	20	16	4	60	40
Adonitol	20	2	18	20	80
Raffinose	20	16	4	60	40
Salicin	20	2	18	20	80
Arginine	20	18	2	80	20

Source: Authors

mediated by which resistance genes are transferred via transposons from one generation to another. Resistance could also be due to rampart use, misuse or abuse of these essential antimicrobial agents.

Since rats are disease reservoirs, humans should make sure that all food is securely covered or stored to stop rodents from contaminating the food. If correct management and hygienic conditions are not taken, *Klebsiella* infections will continue to rise. House rats consume our food products in their struggle to survive, including rice, garri, beverages, vegetables, etc. Because they carry pathogenic microorganisms that can infect humans negatively, proper precautions should be taken to reduce, if not completely eliminate, their interaction with environment. Rats are an important source of zoonotic infections that cause illness and mortality in humans. This is particularly problematic in the school environment with regard to rat-associated health risks because the

Table 4. Antimicrobial susceptibility and resistance of *Klebsiella* isolates commonly use Antimicrobial agents.

S/N	Antibiotics	Drug conc (µg)	Number of susceptible organisms (%)	Number of resistant organisms (%)
1	SXT	30	6(30)	14(70)
2	CH	30	4(20)	16(80)
3	SP	10	16 (80)	4(20)
4	CPX	30	20(100)	0(0)
5	AM	30	6(30)	14(70)
6	AU	10	4(20)	16(80)
7	CN	30	2(10)	18(90)
8	PEF	30	20(100)	0(0)
9	OFX	10	20(100)	0(0)
10	S	30	8(40)	12(60)

SXT- Cotrimoxazole , CH-Chloramphenicol , SP- Sparfloxacin , AM- Amoxacillin , AU-Augmentin , CN- Gentamycin , PEF- Pefloxacin , OFX- Ofloxacin , S- Sreptomycin, CPX-Ciprofloxacin.
Source: Authors

Table 5. Resistant pattern of *Klebsilla* Isolates.

Isolate number	Resistant pattern	Number of resistant antibiotic
1	AM, AU, CH, CN, SP, S, SXT	7
2	AM, AU, CH, CN, SP, S, SXT	7
3	AM, AU, CH, CN, SP, S, SXT	7
4	AM, AU, CH, CN, SP, S, SXT	7
5	AM, AU, CH, CN, S, SXT	6
6	AM, AU, CH, CN, S, SXT	6
7	AM, AU, CH, CN, S, SXT	6
8	AM, AU, CH, CN, S, SXT	6
9	AM, AU, CH, CN, S, SXT	6
10	AM, AU, CH, CN, S, SXT	6
11	AM, AU, CH, CN, S, SXT	6
12	AM, AU, CH, CN, S, SXT	6
13	AM, AU, CH, CN,SXT	5
14	AM, AU, CH, CN,SXT	5
15	AU, CH, CN	3
16	AU, CH, CN	3
17	CN	1
18	CN	1

AM-Amoxacillin, AU-Augumentin, CH-Chloramphenicol, CN-Gentamycin, SP-Sperfloxacin, S-Sreptomycin, SXT- Cotrimoxazole
Source: Authors

University community provides a suitable habitat for rats, leading to close contact between rat and people and potentially zoonotic disease transmission. Some species of house rat like *Mastomys natalensis* has been associated with dangerous viral zoonotic infection such as *Lassa* fever.

Klebsiella infection remains among those infectious diseases of potential serious threat to public health because they attack the host when the immune system is compromised (Ogbole et al., 2022).

Zhong et al. (2020) reported in a study conducted in China that capsular serotyping of *K. pneumonia* have shown to depict important hyper virulent serotypes (K1, K2, K5, K20 and K57) of the bacteria. The hyper virulent seroptypes are the overlapping serotypes observed in both animals and humans (Zhong et al., 2020).

Therefore, capsular serotyping or genotyping is further required to establish the particular serotypes isolated or phenotypically characterize in this study. Hence, molecular study is required to detect resistance genes in these isolates. Finally, this study might have shown zoonotic importance of *K. pneumonia* and *K. Oxytoca* further study on serotyping should be carried out which is one of the limitation of our study for proper characterization of the organism within the study area.

Conclusion

This study shows that *Klebsiella* species exist in rats in the main Campus of University of Abuja. Out of 100 samples analyzed 20(20%) were tested positive to *Klebsiella* and among them, some species were found to be *K*. *pneumoniae* and *K*. *oxytoca* and the percentage of the occurrence of *Klebsiella* specie found are 12(12%) for *K. pneumonia* and 8(8%) for *K. oxytoca*. This prevalence is a thing of concern to public health because rodent has been part of our environment daily. The increasing trend of antibiotic resistance should be of immense public health concern. Proper legislation is needed to control *Klebsiella* infection within the environment.

RECOMMENDATION

More research should be done on animals that live with rats such as giant African rat, mouse, guinea pigs, squirrels, hares and shrews because they are carriers of diseases using advance technology. More drugs should also be used for antimicrobial susceptibility testing because of their increasing growth and resistance of the organism like *Klebsiella* species. Studies should include other microorganisms found in rats in other geographic location. There is need for serious public health awareness of this bacterial infection and its zoonotic signs.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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