


An Investigation of Potential Health Risks from Zoonotic Bacterial Pathogens Associated with Farm Rats

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ABSTRACT

BACKGROUND: The 21st century has seen a wide range of diseases resulting from zoonotic infections, of which bacterial infections have led to outbreaks of food-borne diseases.

AIM: The study looks at bacterial pathogen carriage by farm rats and their antimicrobial susceptibility, with the view of providing insights for antimicrobial surveillance.

METHOD: Farm rats of *Rattus rattus* species were randomly collected alive from farms in Al-Ahsa using food baits. They were anaesthetized with urethane within 4 h of collection and were unconscious for the collection of samples. Basic bacteriological culturing methods were used for culturing of bacterial isolates on selective media while the Vitek 2 compact automated system (BioMerieux, Marcy L'Etoile, France) was used for bacteria identification and antimicrobial susceptibility test. Obtained data were analysed using chi-square and paired *t*-test with significant difference between sensitive and resistance to antimicrobial susceptibility taken at $P < .05$.

RESULTS: Isolated Gramme-negative pathogenic bacteria included strains of *Escherichia coli*, *Pseudomonas oryzihabitans*, strains of *Pseudomonas aeruginosa*, and *Salmonella*. For the Gramme-positive bacteria, 4 strains of *Staphylococcus aureus* were encountered. Other Gramme-positive bacteria were coagulase-negative *Staphylococcal* species (CoNS) as well as *Staphylococcus lugdunensis*. There was a 100% resistance to the penicillins and a high resistance to imipenem (71%) by the *Staphylococcal* isolates. Resistance was also high against the β -lactams by the Gramme-positive bacteria isolates. For the Gramme-negative bacteria, there was a higher than 50% resistance by the isolates against the following antibiotics: ampicillin (78%), amoxicillin/clavulanic acid (67%), cefotaxime (77%), ceftazidime (67%), cefepime (78%), norfloxacin (67%), nitrofurantoin (67%), and trimethoprim/sulfamethoxazole (78%).

CONCLUSION: The results showed high antimicrobial resistance that will need monitoring for control of spread from farm rats to humans.

KEYWORDS: Farm rats, bacterial pathogens, multidrug resistance

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Introduction

The 21st century has witnessed the emergence of many high-profile diseases all of which have been of immense public health concerns. According to the World Health Organization (WHO)¹ report, many of these diseases are of zoonotic origins. The report placed 60% of all recognized human infections as well as 75% of emerging diseases, which have affected humans in the past decade to have originated from animals.¹ In another report, it was highlighted that a wide range of animals which could be domestic or wild are carriers of these diseases.² The associated risk factors for contacting these diseases include close contact by workers in agricultural and livestock industry with infected animals. There is a wide range of diseases resulting from zoonotic infections and includes those of bacterial infections. The WHO² lists some zoonotic bacterial disease causing agents to include *Salmonella*, *Escherichia coli* *Campylobacter* among other bacteria species. Animal reservoirs

are usually responsible for the spread of these infections some of which are rodents belonging to about 220 species.³ Contact with these rodents is also a risk factor for transmission that could subsequently lead to severe infections in humans.⁴ It is also reported that rodents on farms have been linked to the cause and major spread of diseases between humans and animals.⁵ The reasons for such spread of disease causing pathogens have been attributed to difficulties in excluding or completely eradicating them from farmhouses.

There is a wide list of possible bacterial pathogens that can be spread either directly or indirectly by rodents or their ectoparasites, and they include *E. coli*, *Salmonella*, *Campylobacter*, *Listeria*, among others. Earlier reports demonstrated the carriage as reservoir of *Campylobacter* by mice on farms,⁶ while another report associated *Campylobacter* infections with the presence of rats on farms which increased the risk of introducing *Campylobacter* infection into broiler houses.⁷



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Generally, diseases associated with rat-borne zoonotic pathogens have emerged and re-emerged in Europe some of which had led to outbreaks of infection.^{8,9} Of such are those associated with *E. coli* infections. Generally, humans and rats are natural carriers of *E. coli*.¹⁰ However, variant strains of this bacterium can cause diseases in humans such as the enterohemorrhagic *E. coli* (EHEC/VTEC) with reported disease outbreaks attributed to *E. coli* 0157: H7 from visits to farms.¹¹⁻¹⁵ In a recent report Strand and Lundkvist¹⁶ while expressing the uncertainty of whether *E. coli* can be transmitted from rats to human populations, did suggest that rats from different neighbourhoods be monitored as results from such monitoring could be used as possible warning signs of resistant bacteria strains circulating in the region.

The spread of antimicrobial resistant bacteria strains is a global problem requiring the surveillance and reporting from different geographical regions of the world. It is suggested that antimicrobial susceptibility of bacteria strains in rats could parallel those in humans with such studies serving as indicators of the bacterial strains in the surroundings.¹⁶ However, studies on the role played by farm rats, as potential carriers of bacterial pathogens are scanty. There is a need to bridge this gap in information more so because there are growing concerns of increasing resistance to rodenticides by rats with documentations of geographical spread of resistant mutants.¹⁷ It is of the view that *Rattus* species (*Rattus norvegicus*) the brown rat and *R. rattus* the black rat from rural areas would be carriers of more rat borne-microbes than those in urban areas.¹⁶ While there is an increase in surveillance in Europe, literature is silent on such studies in the region of the present investigation. The present investigation therefore looks into the possible carriage of bacterial pathogen by wild farm rats and the antimicrobial susceptibility pattern of the encountered bacteria isolates. This is with the view of bridging the gap in information in the region of this investigation as well as providing necessary information for possible surveillance.

Materials and Methods

Ethical consideration

Permission for the research was given by the research ethics committee of the College of Medicine, King Faisal University with approval number 2017-03-27. Also, experiments were conducted according to the 'guidelines for ethical conduct in the care and use of animals in research' by the American psychological association.¹⁸ They were trapped according to the humane manner of animal care by the American Veterinary Medical Association (AVMA)¹⁹ guidelines. Animals were anaesthetized throughout the process of sample collection, and surgical procedures were carried out by a trained personnel. Disposal of animals was according to guidelines of research and ethical recommendations of Deanship of scientific research, King Faisal University.

Site and the collection of farm rats. The study was in Al-Ahsa located in South east of Saudi Arabia. Al-Ahsa Oasis is a large area with lots of surrounding villages in the Eastern Arabian Peninsula. The economic history of the region is associated with agriculture with broad agricultural activities which includes the growing of diversified crops and fruits such as high-quality dates. Rats of *Rattus rattus* species were collected from different locations around farms in villages at Al-Ahsa with the help of their owners in 2018. Farms were selected randomly but only 3 of the 5 selected farms agreed to help with the trapping of the rats. It was therefore ensured that farm workers did not handle the trapped animals to prevent any associated risks of disease transmission.

Two rats were therefore collected from each farm and used for the investigation. They were trapped using food baits and transported alive in covered rat cages to the animal house of College of Medicine in accordance with recommended guidelines.²⁰⁻²² And were identified by Biological Sciences Department of College of Science, King Faisal University, Al-Ahsa. They were anaesthetized with urethane within 4 h after they were brought from the farms for the collection of samples.²³

Sample collection and microbial culturing

While the animals were unconscious on sterile surgical beds, nasal and rectal swabs were collected with sterile cotton swabs and inoculating loops. All samples were collected under septic conditions, inoculated separately into nutrient broth (NB) and cultured aerobically for 24 h at 37°C. Post collection of nasal and rectal swabs, rats were sacrificed by cervical dislocation for the collection of nasal tracheal swab samples and a loopful of faecal samples from the rectum. Collected sample were inoculated into the NB and incubated aerobically at 37°C for 24 h. A loopful of the resultant overnight growth were each plated out on Blood, MacConkey, *Salmonella* and *Shigella* agar (Oxoid) and incubated aerobically at 37°C for 24 h. Pure colonies of isolated bacteria cultures were used for the identification and antimicrobial susceptibility test.

Bacteria identification and antimicrobial susceptibility test. Bacteria isolates were identified with the Vitek 2 compact automated system (BioMerieux, Marcy L'Etoile, France) according to the manufacturers' guidelines. The Gramme-negative (GN) cards were used for the GN organisms, while the Gramme-positive (GP) cards for the GP isolates. The minimum inhibitory concentrations (MICs), antibiotic susceptibility, and resistance patterns for the isolates were determined with the Vitek 2 compact automated system using the AST cards. The GN (AST-N204) cards had the following antibiotics: ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, fosfomycin, nitrofurantoin, and trimethoprim/sulfamethoxazole. While the GP

Table 1. Distribution of bacterial species encountered in the rats and their site of entrapment.

BACTERIA ISOLATE	VILLAGE FARM 1		VILLAGE FARM 2		VILLAGE FARM 3	
	RAT 1.1	RAT 1.2	RAT 2.1	RAT 2.2	RAT 3.1	RAT 3.2
<i>S. aureus</i> 1 ^a	X	X				X
<i>S. aureus</i> 2 ^a	X	X	X	X		
<i>S. aureus</i> 3 ^a			X	X		
<i>S. aureus</i> 4 ^b					X	X
<i>S. lugdunensis</i> 1 ^b	X	X				
<i>S. lugdunensis</i> 2 ^b					X	X
OCoNS isolate ^c	X	X	X	X	X	X
<i>E. coli</i> 1 ^b	X	X				
<i>E. coli</i> 2 ^b			X	X		
<i>E. coli</i> 3 ^a		X			X	X
<i>E. coli</i> 4 ^c	X		X	X	X	X
<i>P. aeruginosa</i> 1 ^b		X	X			
<i>P. aeruginosa</i> 2 ^a			X	X	X	
<i>P. oryzihabitans</i> ^a	X	X			X	X
<i>Salmonella</i> sp. 1 ^a	X	X			X	X
<i>Salmonella</i> sp. 2 ^a		X			X	
<i>P. mirabilis</i> 1 ^c	X	X	X	X	X	X
<i>P. mirabilis</i> 2 ^c		X		X		X
<i>P. mirabilis</i> 3 ^c	X		X	X	X	
Unidentified ^c	X	X	X	X	X	X

Abbreviations: *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; OCoNS, other coagulase-negative *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. oryzihabitans*, *Pseudomonas oryzihabitans*; *P. mirabilis*, *Proteus mirabilis*.

a= isolates common to two farms.

b= isolate associated with one farm.

c= isolates common to all farms.

(AST-P586) cards consisted of the following antibiotics: benzylpenicillin, ampicillin, amoxicillin/ sulbactam, oxacillin, cefotaxime, cefuroxime axetil, imipenem, gentamicin high level (synergy), streptomycin high level(synergy), levofloxacin, moxifloxacin, erythromycin, clindamycin, quinpristin/dalfopristin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, nitrofurantoin, and trimethoprim/sulfamethoxazole.

Statistical analysis

Data are presented as percentages of both sensitive and resistance strains with respect to isolates susceptibility. Statistical analysis was done using GraphPad Prism 8.2 version. Paired *t*-test was used to assess the significant difference between sensitive and resistant susceptibility of the isolated organisms. Also, Spearman's rho correlation coefficient was used to compare the relationship in antimicrobial susceptibility among the isolates with significant difference taken at $P < .05$.

Results

The results focused on encountered bacterial species that are potential pathogens. The distribution of isolated bacteria and the associated Farm rat is shown in Table 1. There were commonalities and differences in the bacterial strains (Table 1). Among the GNs were 4 *Escherichia coli* (*E. coli*) strains labelled as *E. coli* isolates 1-4. Also encountered was *Pseudomonas oryzihabitans*, 2 strains of *Pseudomonas aeruginosa* labelled as *P. aeruginosa* isolates 1 and 2. Other isolates were species of *Salmonella* labelled isolates 1 and 2 and strains of *Proteus mirabilis*. For the GP bacteria, 4 strains of *Staphylococcus aureus* labelled as *S. aureus* isolates 1-4 were encountered in the study. Other GP bacteria were coagulase-negative *Staphylococcal* species (CoNS) labelled as isolates CoNS 1, CoNS 2, and OCoNS as well as *Staphylococcus lugdunensis*. All isolates showed varying levels of resistance to the antibiotics against which they were tested and the results are presented in Tables 2 and 3. Of the 21 listed antibiotics on the GP card, results were given by the

Table 2. Showing the antimicrobial susceptibility of potential pathogenic Gramme-negative bacterial isolates.

ANTIBIOTICS	BACTERIAL ISOLATES											
	E. COLI ISOLATE 1	E. COLI ISOLATE 2	E. COLI ISOLATE 3	E. COLI ISOLATE 4	P. ORYZIHABITANS	P. AERUGINOSA ISOLATE 1	P. AERUGINOSA ISOLATE 2	SALMONELLA SP. ISOLATE 1	SALMONELLA SP. ISOLATE 2	PROTEUS MIRABILIS 1	P. MIRABILIS 2	P. MIRABILIS 3
Ampicillin	R	R	R	R	R	S	R	R	S	S	S	S
Amoxicillin/ clavulanic acid	S	S	R	R	R	S	R	R	R	S	S	S
Piperacillin/ tazobactam	S	S	R	S	S	S	R	S	R	S	S	S
Cefotaxime	S	R	R	R	R	S	R	R	R	R	I	I
Ceftazidime	S	R	R	R	R	S	R	S	R	S	S	S
Cefepime	S	R	R	R	R	S	R	R	R	S	S	S
Ertapenem	S	S	S	S	R	S	R	N	R	R	S	S
Imipenem	S	R	S	R	S	S	S	N	S	S	S	S
Meropenem	S	R	S	S	R	S	S	N	S	S	R	S
Amikacin	S	R	S	S	R	S	S	R	S	R	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	R	R	S	S	S	S	S	S	S	S	S
Norfloxacin	S	R	R	R	R	S	R	S	R	R	S	S
Fosfomycin	S	R	S	S	S	S	R	S	R	S	S	S
Nitrofurantoin	R	S	R	R	S	S	R	R	R	S	S	S
Trimethoprim/ sulfamethoxazole	R	R	R	S	R	R	R	S	R	S	S	S

Abbreviations: R, resistant; S, sensitive; I, intermediate; N, not given.

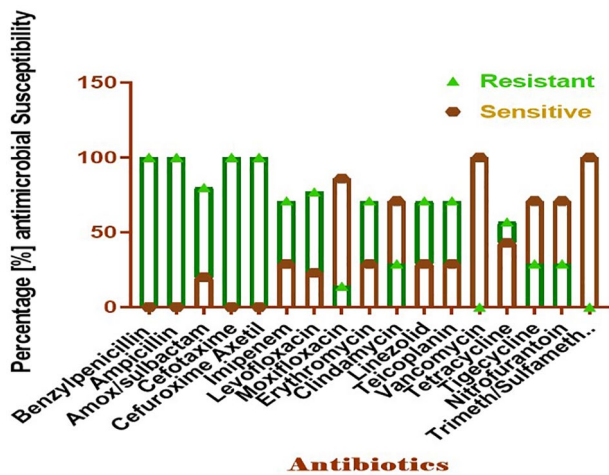


Figure 1. Showing antimicrobial susceptibility of Gramme-positive isolates against test antibiotics.

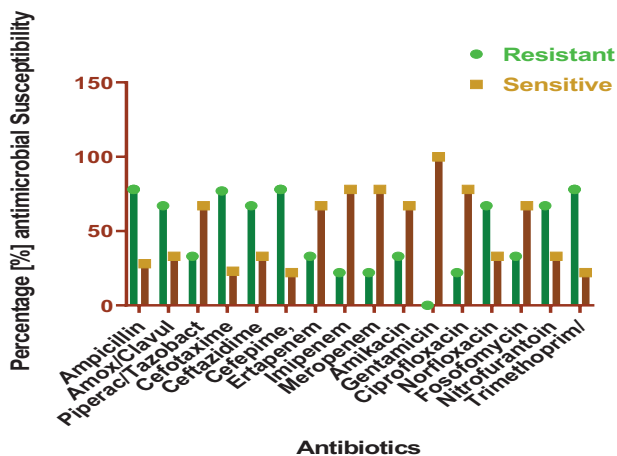


Figure 2. Showing antimicrobial susceptibility of Gramme-negative isolates.

Vitek 2 compact automated system (BioMerieux) for 17 of them, and data were analysed based on these 17 antibiotics.

For the GP bacterial isolates, an overall high antimicrobial resistance by the isolates against 11 of the 17 antibiotics was seen (Figure 1). All isolates were resistant to the penicillins (100%), with an unusually high resistance to imipenem (71%), a last line antibiotic. Percentage resistance was also high against the β -lactams (cefotaxime and cefuroxime axetil). However, all (100%) of the GP bacteria isolates were sensitive to vancomycin and trimethoprim/sulfamethoxazole (Figure 1). The results showed that there is a correlation between antimicrobial resistance and sensitivity pattern giving correlation coefficient (ρ) = -0.991.

Antimicrobial resistance by the GN bacteria against the tested antibiotics showed all the isolates exhibiting varying degrees of resistance as shown in Figure 2. While there was no 100% resistance by any of the GN bacterial isolates against any of the antibiotics, there was a more-than 50% resistance by this group of isolates against the following antibiotics: ampicillin (78%), amoxicillin/clavulanic acid (67%), cefotaxime (77%),

ceftazidime (67%), cefepime (78%), norfloxacin (67%), nitrofurantoin (67%), and trimethoprim/sulfamethoxazole (78%). All GN isolates were sensitive to gentamicin. Also, obtained results showed that there is a correlation between antimicrobial resistance and sensitivity pattern giving correlation coefficient (ρ) = -0.918.

Percentage antimicrobial resistance was also seen to vary among bacterial strains as shown in Tables 4 and 5. The percentage resistance of the GP isolates ranged from 47% to 65% against the tested antibiotic. The comparison of percentage antimicrobial resistance between species of *Staphylococcus* showed that differences were not statistically significant at $P \leq .05$. However when comparing percentage resistance between bacteria strains, the results varied. Of the 17 antibiotics tested, *S. aureus* isolates 1 and 3 were resistant to 11 (65%) of them as results considered intermediate susceptibility to be resistant. However, *S. aureus* isolate 3 showed intermediate susceptibility to 2 antibiotics that were recorded as resistant. Paired *t*-test comparison in percentage antimicrobial susceptibility among the 2 *S. aureus* strains were statistically significant *P* value of .002 while difference in the other *Staphylococcal* isolate were not statistically significant (Table 4). The 2 *S. lugdunensis* isolates differed in their susceptibility to the tested antibiotics with isolate 1 exhibiting more (47%) resistance than isolate 2 (17.6%). Also, for other CoNS isolates, there differences to antimicrobial susceptibility were statistically not significant.

Percentage antimicrobial resistance of the GN bacterial isolate ranged from between 6% and 69% (Table 5). Highest resistance was seen among the following isolates: *E. coli* isolates 2 (69%), *E. coli* isolate 3 (63%), *P. oryzihabitans* (63%), *P. aeruginosa* isolate 2 (69%), and *Salmonella* isolate 2 (63%). The results comparing the percentage antimicrobial susceptibility showed differences in percentages of resistance and sensitivity between the bacterial strains to be statistically significant (Table 5).

Discussion

The bacterial isolates and their susceptibility to the tested antimicrobials as seen in the present findings show that farm rats carry bacterial pathogens which could cause infections in humans either directly or indirectly. The commonalities in some of the isolates could be due to the fact that these farms are interconnected being about 4.6 km apart, while the resistance pattern further points to these bacteria isolates being carriers of multiantibiotic resistant genes. These genes might parallel those in their surrounding environment as had been expressed earlier.¹⁶ The bacteria strains of *E. coli*, *S. aureus*, *P. aeruginosa*, and *Salmonella* species encountered in this investigation are similar to those earlier isolated by some researchers.^{23,24} All of which are listed pathogens isolated from different organs of rats and linked to rodent-borne diseases. Therefore, encountered bacteria pathogen from the farm rats could cause infections in humans through various means.

Table 4. Comparison of percentage antimicrobial susceptibility of Gramme-positive isolates with their *P* values.

BACTERIAL ISOLATE	NUMBER OF TESTED ANTIBIOTICS	% RESISTANT	% SENSITIVE	<i>P</i> VALUE
<i>S. aureus</i> isolate 1	17	11 (65)	6 (35)	.002*
<i>S. aureus</i> isolate 2	17	8 (47)	9 (53)	.54
<i>S. aureus</i> isolate 3	17	11 (65)	6 (35)	.002*
<i>S. aureus</i> isolate 4	17	9 (53)	8 (47)	.54
<i>S. lugdunensis</i> 1	17	8 (47)	9 (53)	.54
<i>S. lugdunensis</i> 2	17	3 (18)	14 (82)	.000**
CoNS isolate 1	17	10 (59)	7 (41)	.07
CoNS isolate 2	17	8 (47)	9 (53)	.54

*Represents significant difference between resistant and sensitive isolates at $P < .05$.

**Represents highly significant difference.

Table 5. Comparison of percentage of antimicrobial susceptibility for Gramme-negative isolates with their *P* values.

BACTERIAL ISOLATE	NUMBER OF TESTED ANTIBIOTICS	% RESISTANT	% SENSITIVE	<i>P</i> VALUE
<i>E. coli</i> 1	16	3 (19)	13 (81)	.00*
<i>E. coli</i> 2	16	11(69)	5 (31)	.001*
<i>E.coli</i> 3	16	10 (63)	6 (37)	.008*
<i>E.coli</i> 4	16	8 (50)	8 (50)	1.00
<i>P. oryzihabitans</i>	16	10 (63)	6 (37)	.008*
<i>P. aeruginosa</i> 1	16	1 (6)	15 (94)	.000*
<i>P. aeruginosa</i> 2	16	11 (69)	5 (31)	.0001*
<i>Salmonella Sp.</i> 1	16	6 (38)	10 (62)	.015*
<i>Salmonella Sp.</i> 2	16	10 (63)	6 (37)	.008*
<i>P. mirabilis</i> 1	16	4 (25)	12 (75)	.0000**
<i>P. mirabilis</i> 2	16	2 (13)	14 (87)	.0000**
<i>P. mirabilis</i> 3	16	1 (6)	15 (94)	.0000**

*Represents significant difference between resistant and sensitive isolates at $P < .05$.

**Represents a highly significant difference between number of antibiotic sensitive to against the number resistant to.

Generally, rodent-borne diseases can be grouped into 2 main categories.²⁴ In the first category, there are diseases which can be transmitted directly either through bites or by inhaling germs from the faeces of rats. The second category are diseases which can be transmitted from rats to humans indirectly by arthropod vectors. There is also the transmission of bacterial pathogens through the eating of improperly cooked contaminated foods.⁴

All the isolated bacteria in this investigation could cause infections in humans in one or more of the listed categories. One of the isolated bacteria in this study is *S. aureus*, a bacterium that has been linked with infections resulting from rat bites.^{16,25} *S. aureus* colonizes the mucous membranes of rats

and is a potential risk factor in causing infections in humans through bites with the bacterium being subsequently translocated to deeper tissues.^{25,26} The susceptibility to antibiotics as seen in this study could be pointers to the strains in the surrounding farms that is worth taking into consideration in regional antimicrobial-resistance surveillance.¹⁶ Generally, *S. aureus* colonizes the human epithelia and is capable of causing from life-threatening bacteremia to septicemia.²⁷ Those isolated in this study were resistant to the penicillins as well as exhibiting a high resistance against the β -lactams. The isolation of methicillin-resistant *S. aureus* (MRSA) from the respiratory track of rats had also been reported.²⁸ Such findings are detrimental to public health as the global problem of

difficult-to-treat bacterial infections, of which MRSA is one, continues to be a threat in the treatment of patients.²⁹⁻³¹ There is the possibility that the antimicrobial resistance as seen among *S. aureus* and CoNS in this study could parallel those in humans thus highlighting the need for close monitoring because they could be warning signs for urgent control measures as earlier expressed.¹⁶

In this study, bacterial isolates that could cause disease by food contamination in humans are *E. coli* and *Salmonella* species. *E. coli*, though a normal microbial gut flora, does have pathogenic serotypes (O157: H7) and had been associated with disease outbreaks of food-borne pathogenic haemorrhagic colitis. The encountered strains in this study highlights the possibility of food-borne infection risk that could result from improperly cooked contaminated farm produce. Similar findings on the isolation of *E. coli* as well as its pathogenic serotypes from wild rodents had been reported by researchers.^{23,32} Also, *Salmonellosis* disease linked to certain serotypes of *Salmonella* is listed by WHO as one of the most commonly distributed food-borne diseases with animals in the wild listed as reservoirs while the Centre for Disease Control (CDC) reported Salmonellosis to be one of the diseases that could be transmitted by rodents.^{2,33} As food poisoning resulting from both *E. coli* and *Salmonella* had been reported in the past in certain regions of Saudi Arabia, there will be the need for the monitoring of farm rats in control measures for the prevention of spread of multidrug-resistant pathogenic bacteria through food contamination.³⁴

Other GN bacterial pathogens encountered in this investigation were *P. aeruginosa* and *P. oryzae* both of which can cause diseases in humans. *P. aeruginosa* is attributed to be one of the most antibiotic resistant bacterium associated with nosocomial infections with the possibility of infections resulting from contamination.³⁵⁻³⁷ *P. oryzae* reported to be CDC group Ve-2 was rarely implicated as human pathogen due to the fact that it is a soil and saprophytic organism. This bacterium has however been more recently associated with diseases in immunocompromised patients.³⁸ With outbreaks of *P. oryzae* Pseudobacteremia resulting from contaminated hospital equipment, there is the possibility of this saprophytic GN bacteria being an opportunistic pathogen in humans as a food borne pathogen as earlier expressed.^{39,40}

Conclusion

The farm rats investigated in this study are shown to be potential carrier of bacterial pathogens. Also the isolated bacteria were found to be multidrug resistant.

Study limitations

Sample size was small due to the reluctance by the owners of the farms to be part of the research.

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Author Contributions

L.B.E. contributed to the research concept, laboratory experiments, data analysis of results and article write-up, and final preparation of report. Y.A.-M., F.A.-M., M.A.-B., S.A.-K., and N.A.-E. contributed to the field collection of samples, laboratory experiments, data analysis of results. All researchers reviewed the write up, contributed in the search for and updating of literature.

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