Original Research Article

Risk factors and Antimicrobial resistance of *Bacillus cereus* isolated from bovine raw milk and fecal matter from dairy farms in Khartoum Bahri locality-Sudan.

ABSTRACT

This study was conducted in Khartoum Bahri locality during the period from 2020 to 2021. A total of 40 random samples of raw milk and fecal matter were collected from different dairy farms in Bahri locality with the objectives to isolate *Bacillus Cereus* organism and to illustrate the resistance pattern of antimicrobial to confirm *B. cereus*. A questionnaire was designed to show the cleanness, mastitis and fecal matter removal status of the farms. Samples were examined for the prevalence of *B. cereus*. Results of the bacteriological methods were confirmed using PCR. The data showed that *B. cereus* was isolated from 9(45%), 15(75%) of the raw milk, and fecal matter, respectively. In farm (1) the prevalence rate of *B. cereus* in raw milk was 4(20%) which was the highest among other farms, 2(10%), 2(10%), 1(5%) in farm2, farm3 and farm 4, respectively. The isolated organisms were found to be highly resistant to Ampicillin\ Sulbactam, Cotrimoxazole, Cefotaxime, Cloxacillin, Colistin and Ceftriaxone and sensitive to Cephalexin, Tetracyclin, Ciprofloxacin, Levofloxacin, streptomycin, Roxithromycin, and Gentamicin.

Key words: *Bacillus cereus*, Antimicrobial susceptibility, PCR.

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INTRODUCTION

Foodborne pathogens have the main concern in public health and food safety. *Bacillus cereus* food poisoning is one of the most important foodborne pathogens worldwide (Ceuppens*etal*., 2013). *B. cereus* is an anerobic spore-forming, heat resistance, Gram–positive bacterium normally disseminated in the environment. It is usually isolated from the soil, plant materials, raw milk, raw meat and processed meat products (Carline *etal*., 2010). Although *B. cereus* is implicated in many food borne illnesses outbreaks in many countries worldwide, however only a few cases are reported because the symptoms are mostly similar to *Staphylococcusaureus* and

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Clostridium perfringens food poisoning (Arnesen*et al*., 2008; Bottone, 2010; Bennett *et al*., 2013). *B. cereus* has been detected on dairy farms soil, feed, faeces and bedding and in farm milking equipment and wash water (Graham, 1998). Parkinson *et al*. (1999) identified risk factors for *B. cereus* contamination of raw milk, to include season, animal housing, feed type, teat soiling, bedding, manure, and mastitis. The importance of these factors was likely to be due to different farming practices and the ubiquitous nature of *B. cereus*. The general consensus is that contamination of raw milk with *B. cereus* occurs through contamination of the teats, followed by contamination transferring from the teats to harvested milk (Magnusson *et al*., 2007). Mechanisms for teat contamination can occurs through contamination with animal bedding material or contamination with fecal matter following consumption of contaminated feed (TeGiffel*et al*., 2002; Visser*et al*., 2007b) or contamination with farm soil or faeces which are the more important sources for introducing *B. cereus* into raw milk (Christianssone*t al*.,1999; Visser*et al*., 2007a).

Teat cleaning and drying prior to milking have been shown to result in a 96% reduction in *B. cereus* contamination of milk (Magnusson *et al*., 2006). *B.cereus* were found to be resistant to penicillin G and sensitive to oxacillin clindamycin, vancomycin, erythromycin, gentamicin, ciprofloxacin, and ceftriaxone (Shawish and Tarabees, 2017). In Khartoum State Sudan Salih and Rabee (2013) isolated *Bacillus spp*. from 74% of mastitic milk samples, 11% of these isolates were *B. cereus.*

Hence, the objectives of this study were to detect the prevalence of *B.cereus* in raw milk and animal feces, risk factors affecting the prevalence of *B. cereus* in raw milk and to evaluate the antibiotic sensitivity pattern to commonly used antibiotics in dairy farms in Khartoum Bahri locality.

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MATERIAL AND METHODS

Study area:

Stratified random samples were collected from different dairy farms in Khartoum Bahri locality to include Elsielate, Hilate kuku, University of Khartoum farm and University of Sudan farm during the period from 2020 to 2021. The microbiological analysis was carried out in the Faculty of Agriculture laboratories University of Khartoum.

Data collection:

A questionnaire was designed to obtain data regarding the different potential risk factors such as mastitis, cleanliness of udder, manure removal, housing conditions,

and milking hygiene; in addition to other factors associated with contaminations of raw milk with *B. cereus*.

Sampling:

Forty random samples of raw milk and feces were collected from dairy farms in four different locations in Khartoum Bahri Locality;20 samples of raw milk and 20 samples from farm feces.

Preparation of samples:

About 5ml of raw milk were taken from each milk tank directly into sterile plastic container. 5 grams of feces sample were collected directly from the cow rectum using sterile container. The containers were clearly labeled by water proof ink using coding system for each sample. The samples were kept in an ice box and then transferred to the college laboratory.

Bacteriological isolation and identification of *B. cereus*

B. cereus selective agar base/ MYP is the media used to isolate the organism from milk and feces samples. The media was prepared by the addition of egg yolk and Polymyxin B Supplement to reach a final concentration of 100 IU of polymyxin B per ml of medium. After18–24 hrs of incubation at 37 °C in anaerobic conditions, the growth of *B. cereus* was determined by the colonial morphology, precipitation of hydrolyzed lecithin around colonies, and the failure of *B.cereus*to utilize mannitol sugar and degradation of glucose (Friker*et al*., 2008).

Antimicrobial susceptibility test

The antibiotic susceptibility testing was performed using the disc diffusion method according to Quinn *et al*. (1999) and CLSI (2012). The antibiotic discs used contain Ampicillin \ sulbactam (20mg), Co-trimoxazole (25mg), Cefotaxime (30mg), Cloxacillin (5mg), Ceftriaxone (30mg), Cephalexin (30mg), Tetracycline (30mg), Ciprofloxacin (5mg), Levofloxacin (5mg), Roxithromycin (15mg), Streptomycin (10mg), Gentamicin (10mg), Ofloxacin (5mg), Amikacin (30mg). The diameter of inhibition zone formed around each disk was measured using Vernier caliper. According to the diameter of the inhibition zone the result was classified as moderate, susceptible and resistant using standardized table supplied by Quinn *et al* (1999) and CLSI (2012).

DNA extraction and polymerase chain reaction

Prior to DNA extraction bacterial cultures were grown on nutrient agar and incubated at 37cº for 18-24hr for preparation of DNA for PCR screening. DNA extraction was

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prepared according to (Knebelsberger andStöger, 2012). DNA extraction, preservation, and amplification were conducted according to Methods in Molecular Biology (2012). Bacterial colonies were suspended into 200µl 1×PBS, then add 500µl of lysis buffer to the suspended colonies and incubate at RT for 10 minutes. Add 700µl of binding buffer to the lysate and gently mix well, and transfer 600µl mixed solution to spin column. and centrifuge at 12000 rpm for 30sec, remove the flow through and repeat binding step. Add 500µl wash buffer A without wetting the rim, and centrifuge at 12000 rpm for 30sec. The flow was removed and 500µl wash buffer B was added without wetting the rim, centrifuged at 12000 rpm for 30sec. The flow was removed and centrifuged at 2000rpm for 1min to dry the membrane completely, then place the spin column in a new 1.5 microcentrifuge tube. apply 60µl elution buffer and incubate for 1 min at RT and finally centrifuged at 12000 rpm for 1 min to obtain extracted DNA.

Polymerase Chain Reaction

Identification of *B. cereus* DNA isolates was performed by targeting the 16srRNA gene. DNA 16s region amplification was performed using the primer set 16s (11) (16Sf 5´-AGAGTTTGATCCTGGCTCAG-3; 16SR5´-CTAGGGCTACCTTGTTAC GA-3) according to (Knebelsberger,T&Stöger, I., 2012) DNA Extraction, Preservation, and Amplification Methods in molecular biology (2012). PCR is performed on thermocycler (heatin and cooling carried out by machine) and it involves three main steps: (1) denaturation of DNA template at 94cº _5min and followed 94_1 min (2) annealing of primers at 58cº _1min, (3) extension of DNA molecules at approx 72_2min and final extension at 72_10min hold at 4cº these three steps are repeated for 30cycle to produce lots of copies of the DNA Sequence of interest. the amplified products (PCR products ~1500 pb).

The products of PCR were separated by an electrophoresis device 1.5% agarose gel to determine the fragment size of DNA, and for visualization and purification. Shorter DNA fragments migrated through the gel more quickly than longer ones thus determining the approximate length of a DNA by running it on an agarose gel alongside a DNA ladder (1kb). Detection DNA bands by intercalating dye (ethidium bromide) and detection using UV light source and imaging system, then assessing the quality and yield of DNA by spectrophotometry. Aspectrophotometry involves

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estimation of the DNA concentration by measuring the amount of light absorbed by the sample at specific wavelength.

RESULTS

Milking practices:

According to the questionnaire results, 2(50%) of the respondents used to wash the cows udder before milking. All of the dairy cow owners 4(100%) did not use detergent for cleaning milking utensils, and three-quarters of the dairy farms 3(75%) did not use towels to dry teats and one-quarter (25%) used to dry the udder after washing using the same towel. Half (50%) of milkers did not wash their hands at the beginning of milking. However, all farmers (100%) did not wash their hands between milkings. Regarding manure removal, half (50%) of the dairy farms used to remove their cows' manure weekly, one-quarter (25%) used to remove it daily, and one-quarter (25%) used to remove it monthly. No mastitis was observed in these farms where the study was conducted. Regarding antimicrobial resistance one-quarter (25%) was found to use penicillin and three-quarters (75%) used Tetracycline and Gentamicin to treat the diseases in their animals (Table 1).

Table (1): Milking practices

Comment [L43]: Table 1 - Milking practices.

Milking practices	Good %	Moderate %	Bad %
Wash udder	2(50)	0	2(50)
Drying udder	1(50)	0	1(50)
Using same towel	0	1(25)	3(75)
Use antibiotics	4(100)	0	0
Use detergent	0	0	4(100)
Protective cloth	0	1(25)	3(75)
Wash hands before milking	2(50)	0	2(50)
Manure removal	1(25) daily	2(50) weekly	1(25) monthly
Wash hands between milking	0	0	4(100)

Prevalence of *Bacillus Cereus* in the examined raw milk and feces.

The prevalence of positive *B. cereus* samples in the examined raw milk was 9(45%) out of 20, while it was 15(75%) in feces out of 20.

The prevalence of *B. cereus* was the highest in farm 1 (20%, 25%), followed by farm 3 (10% 20%) and farm 2 (10%, 15%), and the least was in farm 4 (5%, 15%) in milk and feces respectively (table 2).

Table (2): Isolation rates and prevalence of *B. cereus*

Number of farms	Isolation rates (raw milk)%	Isolation rates (feces)%
Farm (1)	4(20)	5(25)
Farm (2)	2(10)	3(15)
Farm (3)	2(10)	4(20)
Farm (4)	1(5)	3(15)
Total	9(45)	15(75)

Comment [L44]: Table 2 - Isolation rates and prevalence of *B. cereus*.

Identification of *Bacillus cereus*

B. cereus isolates were identified using morphological and biochemical tests.In MYP Colonies were crenate, rough, and dry, with a violet or purple-pink background and were surrounded by ring of dense white precipitate. In blood Agar colonies were large, feathery, dull, gray, granular and opaque with a rough matted surface. The isolates produce β heamolysis. On Nutrient Agar at 37°C the colonies formed large, grey, white, granular colonies with wavy edge. Microscopically, the organisms appeared as a large gram-positive rods in singles-pairs with square ends. *B. cereus* is catalase positive (+), oxidase negative (-), and motile in semisolid media. D. glucose is positive (+), starch hydrolysis (+) and endospores appear after staining by malachite green (Figures 1, 2, 3, 4 and Table 3).

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Fig. (1):*B. cereus* on MYP medium

Comment [L47]: Figure 1 - *B. cereus* on MYP medium.

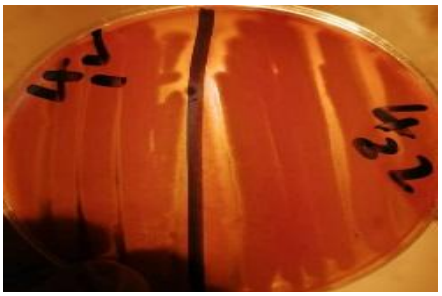

Fig. (2):*B. cereus*' β heamolysis on Blood Agar medium

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Comment [L49]: **Figure 2 -** *B. cereus*' β heamolysis on Blood Agar medium.

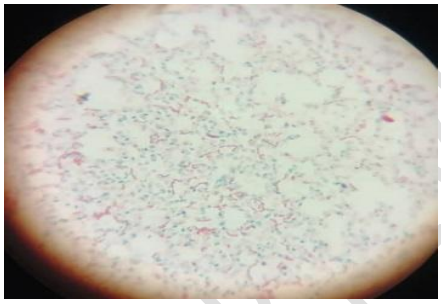

Fig. (3): Stained pores of *B. cereus*

Comment [L50]: **Figure 3 -** Stained pores of *B. cereus*.

Table (3): Biochemical tests of *B. cereus*:

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Number of sample	Glucosfermenation	Starch hydrolysis	Blood heamolysis(β)	Motility test	Endospor staining	Oxidase test	Catalys test	Gram staining
1	+	+	+	+	+	_	+	+
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
5	+	+	+	+	+	_	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	_	+	+
8	+	+	+	+	+	_	+	+
9	+_	+	+	+	+	_	+	+
10	+	+	+	+	+	_	+	+

Antibiotic sensitivity tests of *Bacillus cereus* Isolates

All *B. cereus* isolates were tested for antimicrobial susceptibilities to 14 selected antibiotics. As shown in tables (4 and 5) and figure 4, 5(25%) of the isolates were resistant to Ampicillin/sulbactam, Cefotaxime 5(25%), Cloxacillin 5(25%), Co-

trimoxazole 3(15%) and Ceftriaxone 2(10%) and 1(5%) in milk and feces, respectively. Nearly all isolates were highly sensitive to the remaining antibiotics, such as Ciprofloxacin 5(25%), Levofloxacin 5(25%), Roxithromycin 5(25%), Gentamicin 5(25%), Chloramphenicol 5(25%), Streptomycin 5(25%), Amikacin 5(25%), Ofloxacin 5(25%), Tetracycline 5(25%), Ceftriaxone 3(15%) and 4(20%) in milk and feces, respectively. In addition, it was also found that part of the isolates was moderately resistant to Cotrimoxazole 2(10%). This interpretation was according to the standard of the clinical, and laboratory standards institute disk diffusion method Quinn (1999) and CLSI (2012).

Fig. (4): Clear inhibition zones due to sensitivity of *B. cereus* to antibiotics

Comment [L53]: Figure 4 - Clear inhibition zones due to sensitivity of *B. cereus* to antibiotics.

Table 4 : Antibiotics resistant of *B.cereus* isolated from raw milk (n= 20)

Comment [L54]: Table 4 -Antibiotics resistant of *B.cereus* isolated from raw milk (n= 20).

Name of antibiotics	Sensitive %	Intermediate %	Resistance
Ampicillin/sulbactam20mg	0	0	5(25)
Co-trimoxazole 25mg	0	2(10)	3(15)
Cefotaxim 30mg	0	0	5(25)
Cloxacillin 5mg	0	0	5(25)
Ceftriaxone 30mg	3(15)	0	2(10)
Tetracyclin 30mg	5(25)	0	0
Ciprofloxacin 5mg	5(25)	0	0
Levofloxacin 5mg	5(25)	0	0
Roxithromycin 15mg	5(25)	0	0
Gentamicin 10mg	5(25)	0	0
Chloramphenicol 30mg	5(25)	0	0
Streptomycin 10mg	5(25)	0	0
Ofloxacin 5mg	5(25)	0	0

Comment [L55]: Sulbactam 20mg

Amikacin 30mg	5(25)	0	0

Table 5: Antibiotics resistance of *Bacillus cereus* isolates from feces (n= 20)

Comment [L56]: **Table 5 -** Antibiotics resistance of *Bacillus cereus* isolates from feces (n= 20).

Name of antibiotics	Sensitive %	Intermediate %	Resistance %
Colxacillin 5mg	0	0	5(25)
Roxithromycin15mg	5(25)	0	0
Ciprofloxacin 5mg	5(25)	0	0
Tetracyclin 30mg	5(25)	0	0
Chloramphenicol 30mg	5(25)	0	0
Levofloacin 5mg	5(25)	0	0
Gentamicin 10mg	5((25)	0	0
Streptomycin 10mg	5(25)	0	0
Ceftriaxone 30mg	4(20)	0	1(5)
Ofloxacin 5mg	5(25)	0	0
Ampicillin 20mg	0	0	5(25)
Co-trimoxazole 25mg	0	2(10)	3(15)
Cefotaxime 30mg	0	0	5(25)
Amikacin 30mg	5(25)	0	0

Polymerase chain reaction

The 16SrRNA gene sequence of the isolats was about 1500 bp in length. PCR results showed the presence of bacterial genes, and based on chemical tests and morphological characteristics, the presence of *Bacillus cereus* can be predicted (Figure 5).

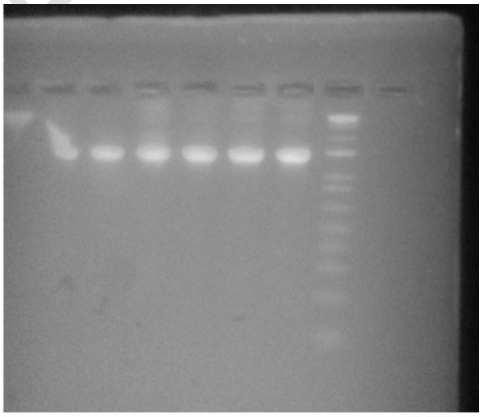

Fig. (5): Amplified Col fragments on agarose gel DNA (lader right)

Comment [L57]: **Figure 5 - Amplified Col fragments on agarose gel DNA (lader right).**

PCR products with multiple bands (1500pb)

DISCUSSION

The present study was conducted to detect the risk factors on raw cow milk and fecal matter samples to detect the prevalence of *B.cereus*and its antimicrobial resistance. The prevalence of *B. cereus* was 45% in raw milk and 75% in feces. This result was higher (38.80%) than the prevalence rate reported by Abraha*et al*. (2017) in Haramaya district in Ethiopia in raw cow's milk, and the isolation rate of *B.cereus* was 27% from raw milk reported in Abidjan by Yobou et al. (2014).On the other hand, a higher prevalence rate of *B. cereus* (66.6%) from raw milk samples was recorded in a previous study carried out in Egypt (Organji*et al*., 2015). This variation could be due to the variation in sample size and the improper sanitation measures during collection, and handling of milk which constituted the main cause of the high prevalence rate of *B. cereus* in raw milk samples (Abraha*et al*., 2017).

In this study, the isolation rate from feces was 75%. Higher results (92.9%) were also recorded in a study conducted by Choi *et al*. (2014) in Beijing, China.

In the present study, the prevalence of *B. cereus* in raw milk was the highest in farm 1, 4 (20%), followed by farm 2, 2(10%) and farm 3, 2(10%), and the least was in farm 4, 1(5%). This variation may be due to the differences in farm management systems between the different farms and the good hygienic practices may be better practiced at farm 4 compared to other dairy farms. Improper sanitation during collection and handling of milk, may be one of the main causes of this high prevalence rates of *B. cereus* in farm (1), farm (2) and farm(3), because milkers on farm (1) used to use one towel for all cows. The reuse of one towel for cleaning may result in recontamination of the udder. The manure removal from the farm was not performed daily and this might be the major reason for the contamination of udder and raw milk. Maintaining the sanitary condition of milking area is an important factor for the production of good quality milk. Therefore, it's likely that raw milk might be contaminated from soil environment.

As observed during the present study, half 2(50%) of the respondents washed the cows udder before milking, however all of the dairy cow owners 4(100%) did not use detergent for cleaning milking utensils, which may lead to insufficient cleaning and hence could serve as a major cause of milk recontamination. Furthermore,

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4(100%)milkers did not wash their hands betweenmilkings. This malpractice might contribute to the high level of contamination of milk.

The results of PCR showed that all the isolates contained the bacterial gene. Antibiotic-resistant bacteria pose a growing problem of concern worldwide since the bacteria can be easily circulated in the environment. The effectiveness of current treatments and the ability to control infectious diseases in both animals and humans may become hazardous (Normannoet *al*., 2007). As infections and diseases caused by *Bacillus cereus* can lead to death (Lund *et al*., 2000; Dierickt *al*., 2005) so effective antibiotic therapy must be chosen to treat *B. cereus*. The resistance to penicillin and ampicillin as well as to cephalosporin among *Bacillus spp.* has most frequently been observed (Murray *et al*., 2005). In the present study, the *B. cereus* isolates from both milk and manure had higher resistance rates against different antibiotics in the milk and manure, respectively. These results were comparable with those discussed previously by several other authors. Luna *et al*. (2007) reported that 95% of *B. cereus* isolates were resistant to each of penicillin and ampicillin. Moreover, recent research proved that *B.cereus* isolates were resistant to beta-lactam antibiotics (DasilvaFernandeset *al*., 2014; Kim *et al*., 2015; Yibaret *al*., 2017). However, the present study is in line with the results of Drobniewski (2014), Seblewengel 2013) and Agwa, Uzoigwe and Wokoma (2012).

In the questionnaire results 1(25%) of the farms was found to use penicillin and 3(75%) used tetracycline to treat the diseases in their animals.

Conclusion and Recommendations:

From this study we conclude that the prevalence of *B. cereus* was high in raw milk and feces and *B. cereus* sensitive to Tetracycline and Gentamicin.We recommend the use of Tetracycline and Gentamicin for treatment of mastitis, avoiding the prudent use of antibiotics and thermal treatment of raw milk.

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Comment [L64]: 100%) milkers

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