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Research Article
Prevalence of Multidrug Resistant (MDR) Novel *Enterococcus faecium* Strain VDR03 in Broiler Chicken Meat Samples Collected from Dibrugarh Town, Assam (India)

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Abstract
Prevalence of multidrug resistant bacteria (MDR) in hospital wastes and food have scaled up the level of difficulty in treatment of any disease. Broiler chicken meat is consumed by a large section of population making it an essential diet for the non-vegetarians. The current study was made to screen and characterize MDR strains of Enterococcus, methicillin and vancomycin resistant *Staphylococcus aureus* (MRSA and VRSA) in broiler chicken meat collected from various meat shops located in Dibrugarh town of the state Assam, India. A total of 119 *S. aureus* were screened from 20 meat samples collected from 20 different meat shops. Out of those, 34 (28.6%) isolates were confirmed to be MRSA and VRSA each and 16 (13.4%) strains of *S. aureus* showed resistance against both methicillin and vancomycin. They were confirmed by biochemical tests and staining techniques. The isolate showed resistance against maximum number of antibiotics (viz., polymixin B, metronidazole, novobiocin, tetracycline, rifampicin, erythromycin, methicillin and vancomycin) was identified as *Enterococcus faecium* strain VDR03 on the basis of 16S rDNA sequencing. Plasmids that are responsible for antibiotic resistance were found to be of 2 and 13 kbp size. The GC content of the genomic DNA was found to be 30.74%. The thermal point of the isolate was determined to be 73°C and current study showed the presence of MDR strains in broiler chicken meat but the thermal death point of the most potent isolate suggests that cooking of meat for a longer duration of time may prevent MDR infections.

Key words: Multidrug resistant bacteria, MRSA, VRSA, *Enterococcus faecium*, molecular characterization

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Multi-drug resistance (MDR) refers to a condition enabling pathogenic organisms to resist distinct antibiotics of a wide variety of structure and function targeted at eradicating the organism. Many different bacteria now exhibit MDR including staphylococci, enterococci and gonococci. *Staphylococcus aureus* is an opportunistic pathogen often carried asymptomatically on the human body, presence of which in food body indicates poor sanitization (Cuny *et al*., 2010). Increased multi-drug resistance has become an issue of global concern. Approximately 25-40% of the population shows MRSA colonies which was found responsible for skin and soft tissue infection and sometimes causes severe disease such as pneumonia, meningitis, bacterimia, sepsis and pericarditis (Lowy, 1998; Songer and Post, 2005). The MRSA is any strain of *Staphylococcus aureus* that has developed resistance to beta-lactam antibiotics through the process of natural selection, which include the methicillin, oxacillin, dicloxacillin, nafcillin, penicillin, etc (Kwon *et al*., 2006). The *Staphylococcus aureus* strains which show resistance for methicillin may also exhibit resistance against glycopeptides antibiotic vancomycin (Thati *et al*., 2011). High level vancomycin resistance in *S. aureus* has been reported rarely (Gould, 2010). *In vivo* and *in vitro* experiments reported in 1992 by Noble *et al.* (1992) demonstrated that vancomycin resistance genes from *Enterococcus faecalis* could be transferred by horizontal gene transfer to *S. aureus*, making them highly resistant to vancomycin (Noble *et al*., 1992). In 2002, a VRSA strain was isolated from the catheter tip of a diabetic, renal dialysis patient in Michigan (Chang *et al*., 2003). From 2002-2010, ten additional VRSA isolates were reported; eight from United States, one from India and one from Iran (Gould, 2010). In the end of June, 2013, VRSA isolates were reported for the first time in Latin America and in Europe (Melo-Cristino *et al*., 2013). Enterococcal infections are a threat to human health, largely due to the difficulty in eradicating them with antibiotics. Enterococci have gained considerable importance as nosocomial pathogens through their intrinsic and acquired resistance to the antibiotics most commonly administered in human medicine (Shepard and Gilmore, 2002). Antibiotics are also used for animal growth promotion or for treatment and control of animal diseases, this may hasten the appearance of Antibiotic Resistant (AR) bacteria in humans. Moreover, their resistance to extremes in temperature, pH and salinity entails that enterococci can survive in fermented and even cooked meat (Houben, 2003).

Food may serve as a vehicle to disseminate MRSA, VRSA and *Enterococcus* spp. Low degree contamination with *S. aureus* is common in retail meat MRSA and VRSA has been reported in a variety of meats including raw chicken, turkey, pork, veal, beef, mutton, lamb, rabbit, etc (Lee, 2003). Prevalence of MRSA was found upto 17.5% among rural people around Dibrugarh town of India in a pilot survey undertaken by Borkakoty and Biswas in 2007 (ICMR., 2007). But no research was carried out in the region for characterizing pathogenic MDR isolates from broiler meat samples with the evidence of publication, which may be a reason for the prevalence of MRSA and VRSA in the region.

In the present study, effort was made to isolate and characterize MRSA, VRSA and other MDR isolates from broiler chicken meat samples collected from various meat shops located in Dibrugarh town, Assam (India). Further, molecular characterization of the most potent MDR strain was also carried out.

MATERIALS AND METHODS

Chemicals and consumables: All the chemicals used in the study were procured from Merck India Pvt. Ltd. and all the media used were purchased from HiMedia India Pvt. Ltd.

Collection of meat samples: A total of 20 broiler meat samples were collected from 20 different shops located in Dibrugarh town of Assam. Samples were collected in sterile plastic packets (Supplied by HiMedia India Pvt. Ltd.) kept in ice box.

Isolation and screening of methicillin and vancomycin resistant *Staphylococcus aureus* and MDR strains from meat samples: Serial dilution was carried out to the meat samples. *Staphylococcus aureus* was isolated from the meat samples by spread plating the samples from the final dilution tube on Mannitol Salt Agar (MSA). Plates were incubated at 37±2°C for 24 h (Scigenics Orbitec incubator India Ltd.). Yellow color colonies obtained after incubation were sub-cultured on nutrient agar plates to obtain pure culture. Screening of methicillin and vancomycin resistant *S. aureus* was carried out by spread plating over night LB broth ($OD \geq 1.0$) on Muller Hinton Agar (MHA) plates, on which antibiotic discs of methicillin (5 mcg) and vancomycin (30 mcg) (HiMedia India Ltd.) were placed and incubated overnight at 37±2°C. Data obtained after incubation were tabulated and the desired strains were considered for further processing. The MRSA and VRSA were identified by using commercially available biochemical test kits KB004™ (HiMedia India Pvt. Ltd.).

Screening of the most potent strain on the basis of antibiotic susceptibility: The previously screened isolates were re-inoculated on MHA plates containing metropenem (10 mcg), polymixin B (100 units), metronidazole (5 mcg), novobiocin (30 mcg), cefixime (5 mcg), ciprofloxacin (5 mcg), tetracycline (30 mcg), gentamycin (30 mcg), amikacin (30 mcg), rifampicin (5 mcg), erythromycin (15 mcg) and ampicillin (10 mcg) antibiotic discs and were incubated. The strain which could resist the maximum number of antibiotics was considered for further processing.

Identification of the most potent strain: The most potent strain was identified on the basis of Grams staining and biochemical characterization, prescribed by Bergey's manual of systematic bacteriology. The strain was finally confirmed by16S rDNA sequencing.

Identification of the most potent isolate by 16S rDNA sequencing: The DNA was isolated from the best potential bacterial isolate and its quality was evaluated on 1.2% agarose gel on which a single band of high molecular weight DNA was observed. Fragment of 16S rDNA was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1,500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove any contaminants. Fragment of 16S rDNA was amplified. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F (5′-AGAGTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers using BDTv3.1 cycle sequencing kit on ABI3730×1 Genetic Analyzer. Consensus sequence of 1342 bp 16S rDNA was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the NR database of NCBI GenBank database. Based on maximum identity score first 10 sequences were selected and aligned using multiple alignment software program Clustal W™. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 software. The sequence obtained was deposited in NCBI GenBank and an accession number was received. The evolutionary history was inferred using the neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura (1980) 2-parameter method (Felsenstein, 1985). Phylogenetic analysis was conducted using MEGA4 software (Tamura *et al*., 2007).

Molecular characterization of the isolate: Plasmid DNA was isolated from overnight culture of the most potent strain by using osmotic solution (50 mM tris-HCl, 20 mM Na-EDTA, 15% glucose, 0.2 N NaOH, pH-82), SDS solution (1% SDS) and 3 M sodium acetate solution as described by Borah (2012). Size of the plasmid DNA was estimated by comparing with 1 kb DNA ladder (Merck Biosciences Pvt. Ltd.) after resolving the samples in 0.8% agarose gel.

Determination of GC content of the genomic DNA: The DNA was isolated from overnight culture of the strain by using lysis buffer (50 Mm tris-HCl, 20 mM EDTA, 1.25% SDS) and 3 M sodium acetate solution following the protocol as described by Borah (2012). The GC content was determined spectrophotometrically with the help of melting point and using the following formula (Harisha, 2007):

$$GC\ (\%) = K\ (T_m\text{-}69.4)\times 2.44$$

Determination of Thermal Death Point (TDP) of the potent isolate: The TDP was determined by checking the growth of the isolate on LB agar plates after incubating the bacterial broth at a particular temperature for 10 min (Aneja, 2009).

Statistical analysis: All the experiments were performed in triplicate and results were expressed in terms of Mean±SD. Students t-test was performed to see the statistical significance of the data obtained during the experiments.

RESULTS

Methicillin and vancomycin resistant *Staphylococcus aureus* and multidrug resistant Enterococcus bacterial strains were isolated from 20 different broiler chicken samples by performing serial dilution and streak plate method on Mannitol salt agar media. The red colored media turned yellow with bacterial colony growth indicates positive for Staphylococcus strains. A total of 119 bacterial colonies were obtained on mannitol salt agar with characteristic growth, out of which 34 (28.57%) were confirmed to be MRSA and VRSA each. Another 16 (13.44%) colonies were found to be resistant against both methicillin (5 mcg/disc) and vancomycin (30 mcg/disc) antibiotics (Fig. 1). Presence of *S. aureus* was confirmed biochemically by using commercially available test kits KB004™ (HiMedia, India Pvt. Ltd.) and also by various staining techniques. They were found to be positive for urease, arginine utilization, mannitol, sucrose, lactose, arabinose, raffinose, trehalose, maltose fermentation test. All

the 119 isolates were subjected to antibiotic susceptibility test to find out the most potent MDR strain. About 84 (70.59%) isolates were found to be showing resistance against 3 or more number of antibiotics and were considered as MDR strains. The most potent strain (sample ID: B_1) was found to be resistant against 8 different types of antibiotics (viz, polymixin B, metronidazole, novobiocin, tetracycline, rifampicin, erythromycin, methicillin and vancomycin) (Table 1). The strain was identified as *Enterococcus faecium* strain VDR03 (NCBI GenBank accession No. KJ698643) on the basis of biochemical tests and 16S rDNA sequencing. The percentage of replicate trees in the bootstrap tests (500 replicates) was shown next to the branches in Fig. 2.

Table 1: Results of antibiotics test by the most potent isolate

B1	Antibiotics used and their zone of inhibition (mm) (Sample ID)
R	Methicillin (5 mcg/disc)
R	Vancomycin (30 mcg/disc)
18±2	Ampicillin (10 mcg/disc)
13±1	Metropenem (10 mcg/disc)
19±1	Gentamycin (30 mcg/disc)
R	Tetracycline (30 mc/disc)
21±2	Amikacin (30 mcg/disc)
22±1	Ciprofloxacin (5 mcg/disc)
R	Rifampicin (5 mcg/disc)
R	Erythromycin (15 mcg/disc)
R	Polymixin-B (100 units)
R	Metronidazole (5 mcg/disc)
R	Novobiocin (30 mcg/disc)
10±2	Cefixime (5 mcg/disc)

Plasmid DNA of the isolate was isolated. Gel image shows 2 plasmids with molecular weight 2 and 13 kbp (Fig. 3). The GC content of the strain was found to be 30.74%. Thermal death point of the strain was found to be 73°C (Table 2).

DISCUSSION

Worldwide emergence of multidrug resistance among Gram-negative and Gram-positive bacteria has resulted in a

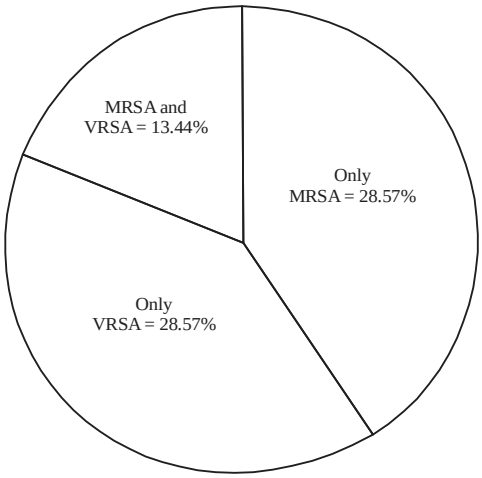

Fig. 1: Percentage prevalence of MRSA, VRSA and *S. aureus* strains showing resistance to both methicillin and vancomycin

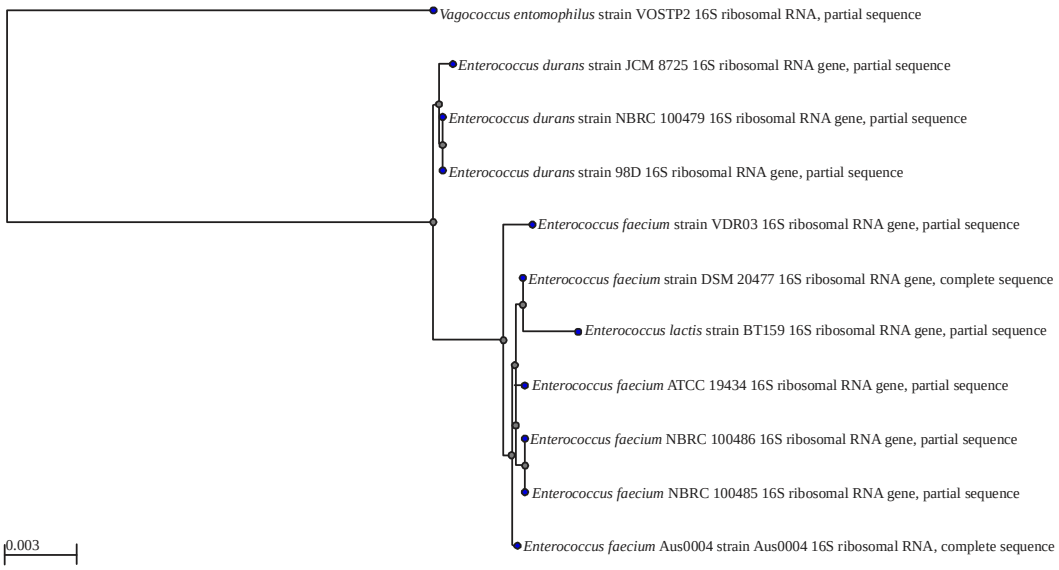

Fig. 2: Phylogenetic relationships of *Enterococcus faecium* strain VDR03 (GenBank accession No. KJ698643) and other closely related *Enterococcus faecium* based on 16S rDNA sequencing. The tree was generated using the neighbor-joining method

Table 2: Result of thermal death point

	Temperatures (°C)													
Growth of bacterial colonies	60	70	71	72	73	74	75	76	77	78	79	80	90	100
Enterococcus faecium VDR03	+++	++	++	+	-	-	-	-	-	-	-	-	-	-

+++: Loxurious, ++: Slow, +: Poor, -: No growth after incubation at 37°C for 24 h

confounding scene in treatment modalities. Bacteria like methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA) and *Enterobacterium faecium* have created a dilemma regarding the appropriate antibiotic therapy to use against them. Infections caused by these bacteria are often difficult to treat. Further, these bacteria can survive for a long time under hospital environment, with increased opportunities for transmission between patients worldwide but recently it is increasingly identified as the etiological agent of infections acquired in community (Mohanty *et al*., 2006). Staphylococcal food poisoning is one of the most common causes of food borne illness due to widespread occurrence of *S. aureus* as well as the ability of many strains to synthesize one or more staphylococcal enterotoxins (Baek *et al*., 2009). *Staphylococcus aureus* is an important cause of a variety of diseases in humans and animals worldwide (Gilot and van Leeuwen, 2004). Several reports suggest that the transfer of *S. aureus* between human and poultry is possible and that the infection of humans by transmission through food and food products contaminated with animal MRSA is very plausible (Roberson *et al*., 1994; Zadoks *et al*., 2000; Lee, 2003; Kaszanyitzky *et al*., 2004).

In the present study, the presence of MRSA, VRSA and MDR *Enterococcus faecium* were confirmed and characterized. Prevalence of 28.57% of MRSA and VRSA each were confirmed in the current study. Data showed 13.44% of *S. aureus* were resistant against both methicillin and vancomycin. Higher contamination rate of MRSA (44%) was found in raw poultry meat in a study conducted by Karmi (2013) in Qena, Egypt. Other studies also reported the levels of MRSA and VRSA in chicken meat and its products as 43% (Lim *et al*., 2010), 52.04% (Shareef *et al*., 2009), 47.2% (Citak and Duman, 2011) and 50% (Lee *et al*., 2001). Moreover, the current study confirms the prevalence of MDR strains upto 70.59% (Fig. 4). The most potent stain was identified as *Enterococcus faecium* strain VDR03 on the basis of 16S rDNA sequencing. It was found resistant against 8 antibiotics (Table 1). Two plasmids with diverge genome size could be isolated from the strain and were suspected to provide antibiotic resistance to the bacteria (Fig. 3). The TDP of the bacteria was found to 73°C, which indicates proper cooking of meat may prevent the contamination of MDR strains from food to human body.

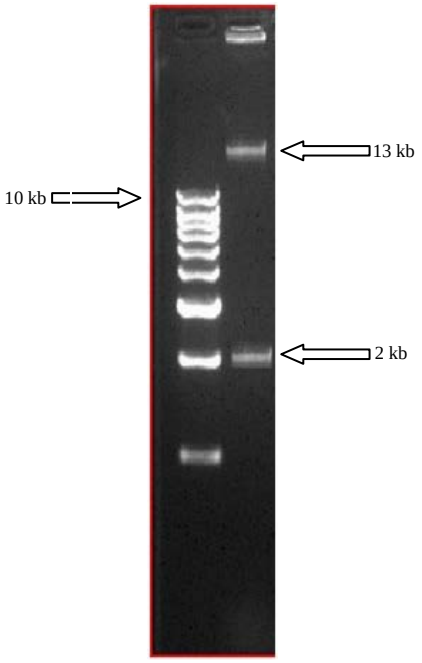

Fig. 3: Gel image of plasmid DNA isolated from the strain

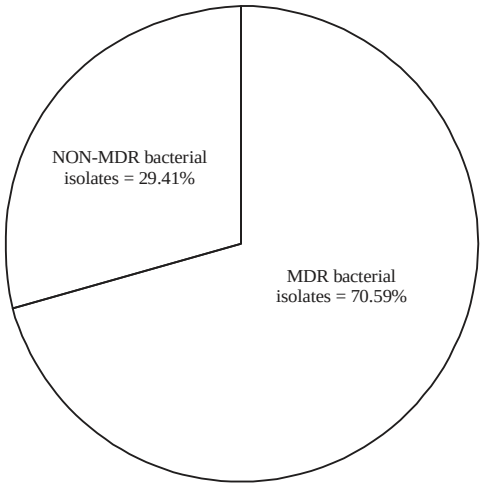

Fig. 4: Prevalence of MDR bacteria in broiler chicken meat

CONCLUSION

Gram positive cocci are among the most frequently isolated bacteria from clinical specimens. Staphylococcus and enterococci are common cause of hospital infections. But

food may also serve as a potential carrier for transmission of these MDR bacteria to humans. The aim of our study was to show the prevalence of MRSA, VRSA and multi drug resistant Enterococcus strains in chicken and the findings revealed the presence as 70.59%. This high value increases our level of concern and thus it becomes a dire need to properly cook the chicken meat before consumption. The presence of *S. aureus* in meat is often attributed to inadequate hygiene during handling by the individuals involved in the production of meat. Poultry meat handled in the cutting section must be stored at low temperature to minimize bacterial growth. Contaminated poultry meat can transfer expressive amounts of *S. aureus* to stainless steels and polyethylene surfaces.

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