SUMMARY

This study was carried out in two dairy farms in Beni-Suef district during the period from September 2014 till April 2016 to investigate the investigate the prevalence, risk factors associated with diarrhea in cow/calf farms, assess the current patterns of antibiotic use and molecular characterization of resistance genes in enteropathogenic bacteria recovered from dairy calves and their environment. The present study was divided into two parts to achieve the above mentioned aims as following:

Part I: The Prevalence and distribution of calf diarrhea in the examined farms:

A structured questionnaire was administered to collect data on calf management, calf diarrhea (frequency, distribution and risk factors), pattern of antibiotic use and biosecurity measures. Samples were collected from both calves (fecal samples) and their surrounding environment (soil, water, swabs from milk buckets, milk samples, swabs from teat apices, swabs from attendant hands, flies, swabs from water devices, swabs from manager and feeding stuffs) using stratified random sampling technique throughout the study period in the investigated farms and then were cultivated for the isolation of diarrhea causing agents, then the bacterial isolates were identified using biochemical and serological techniques. Based on the bacteriological findings the prevalence of calf diarrhea and frequent distribution of enteropathogenic bacteria isolated from both calves and their environment were detected.

The obtained results showed that:

1. The prevalence of calf diarrhea was significantly high in farm (I) compared to farm (II) (36.0 and 26.6%, respectively) at X^2= 35.9; *P*< 0.001, while the percentage of apparently healthy calves were (64.0 and 73.4%, respectively) in both farms (I and II).
2. The frequent distribution of bacteria isolated from diarrheic calves (fecal samples) was significantly high in both examined farms (I and II) (91.8 and 86.2 %, respectively) at X^2=11.49; *P*< 0.009, compared to the apparently healthy ones (31.0 and 26.3%, respectively).
3. The frequency of enteropathogenic bacteria isolated from calves was higher in farm (I) than in farm (II) (93 and 67 bacterial isolates respectively). Moreover, *E. coli* was the most predominantly isolated bacterial pathogen in both farms (I and II) (64.5 and 70.1%, respectively), followed by *Klebsiella* spp., *C. perfringens*, *Shigella* spp. and *Salmonella* spp. in farm (I) (16.1, 11.8, 4.3 and 3.2%, respectively), while in farm (II) *E. coli* followed by *Klebsiella* spp., *Shigella* spp. and *C. perfringens* (16.4, 7.5, and 6.0%). at X^2= 57.57; *P*< 0.001.
4. Serological tests showed that the most detected serogroups from diarrheic calves in farms (I and II) were O_{26}, O_{55} and O_{159} (29.0, 22.6 and 16.1%, respectively) followed by O_{111}, un-typed serogroups and O_{127} (12.9, 9.7, 6.5%, respectively) while $O_{103}$was the least one to be detected (3.2%). Furthermore O_{26} in farm (I) (33.3%) and O_{55} in farm (II) (30.8%) were the most predominant isolated serogroups.
5. The frequent distribution of enteropathogenic bacteria isolated from calves' environment in farm (I) indicated that *E. coli* was the most isolated bacterial pathogen from the different environmental samples (45.3%) as well from the calves (64.5%), followed by *Klebsiella* spp.,

Salmonella spp., *Shigella* spp. and *C. perfringens* (32.0, 10.7, 7.3 and 4.7%, respectively). Whilst the highest percentage of *E. coli* was recovered from soil (66.0%), followed by flies (47.8%), water trough (39.1%), attendant hands (38.5%), and feed manager (30.8%) and the least percentage was recovered from milk bucket (18.8%). *Klebsiella* spp. were recovered in the highest percentage from milk bucket (75.0%), followed by water trough (47.8%), attendant's hands (46.2%), feed manager (23.1%), flies (21.7%) and the least percentage was detected in soil samples (10.6%). Whilst *Salmonella* spp. were recovered from feed samples in the highest percentage (50.0%), followed by flies (21.7%), manager (15.4%), attendant's hands (11.5%) and finally soil sample (10.6%). Meanwhile, *Shigella* spp. were isolated from manager at a percentage of (15.4%) followed by flies, soil, milk bucket, water troughs, and attendant's hands (8.7, 8.5, 6.3, 4.3 and 3.8%, respectively). *C. perfringens* was the least microbial pathogen that was detected in the environment of farm (I) mostly in feed sample (50.0%) followed by manager (15.4%), water trough (8.7%) and soil (4.3%) at X^2= 59.84; P<0.001.

6. From the frequent distribution of bacteria isolated from calves' environment in farm (II) it has been found that *E. coli* was the most prevalent bacterial pathogen (50.3%) followed by *Klebsiella* spp., *Salmonella* spp. and *Shigella* spp. (34.9, 8.1, and 6.7% , respectively). Furthermore *E. coli* was predominantly isolated from water troughs (55.6%) and teat apices (55.3%), followed by attendants hands (51.9%), manager (47.1%), and flies (38.7%). Meanwhile, *Klebsiella* spp. were mainly detected in manager (41.2%), followed by water trough, teat apices, attendant's hands, and flies (40.7, 40.4, 37.0 and 16.1%, respectively). Whilst *Salmonella* spp. were only detected in flies and swabs from attendant's hands at a percentage of (35.5 and

3.7%, respectively). *Shigella* spp. were mostly isolated from feed manager (11.8%) followed by flies, attendant's hands, teat apices and water trough (9.7, 7.4, 4.3, and 3.7%, respectively) at X^2= 56.74; *P*< 0.001.

7. Serological tests showed that *E. coli* O_{55} was the most predominant serogroup to be isolated from farm (I) environment (32.0%) followed by O_{26}, O_{111}, O_{159}, O_{127}, and Un-typed (20.0, 16.0, 16.0, 8.0 and 8.0%, respectively). Moreover attendants' hands and water trough represent a potential reservoir for O_{55} in this farm as it was detected in the highest percentage (50.0 and 44.4%, respectively) followed by milk bucket, manager and soil (33.3% each). Water trough considers the main source of O_{26} (44.4%), followed by milk bucket and soil (33.3% each), then attendants' hands and flies (25.0 and 20.0%, respectively) While, O_{111} was mainly isolated from soil, milk bucket and attendants' hands (33.3, 33.3 and 25.0%, respectively), *E. coli* O_{127} was detected in water trough and flies samples (44.4 and 20.0%, respectively). On the other hand, *E. coli* O_{159} was detected mainly in water trough, flies and manager (44.4, 40.0 and 33.3%, respectively), while un-typed serogroups was mainly detected in feed manager and soil samples (33.3% each).
8. *E. coli* O_{26} was the most predominant serogroup isolated in farm (II) (25.0%) followed by serogroup O_{103}, O_{159} and O_{55} (15.0% each), then serogroups O_{111}, $O_{127}$and un-typed (10.0% each). Furthermore O_{26} was mainly detected in swabs from teat apices, flies, water trough and attendant's hands (40.0, 33.3, 25.0 and 20.0%, respectively). While *E. coli* O_{103} was mostly detected in water trough, teat apices and attendants' hands (25.0, 20.0, 20.0%, respectively). Serogroup O_{159} was detected in feed manager, teat apices and attendant's hands (33.3, 20.0 and 20.0%, respectively).While, *E. coli* O_{55} was mainly detected

in flies, attendant's hands and water trough (33.3, 25.0 and 20.0%, respectively). On the other hand, *E. coli* O_{111} was detected in swabs from teat apices and attendant's hands (20.0% each), O_{127} was detected in flies and water trough (33.3 and 20.0%, respectively), and finally un-typed serogroup was only detected in swabs from feed manager (66.7%).

9. Concerning the serotyping of *Salmonella* spp. in farm (I) the results indicated that *S. enteritidis* was the most frequently detected serotypes from diarrheic calves and their environment (61.1%), followed by *S. kentuky*, *S. typhimurium* and *S. dublin* (22.2 and 11.1, 5.6%, respectively). Moreover, *S. enteritidis* was detected in feed manager and attendants' hands (100.0% each), followed by diarrheic calves, flies and soil (66.7, 40.0 and 40.0%, respectively). While *S. kentucky* was mainly detected in soil, diarrheic calves and flies (40.0, 33.3 and 20.0%, respectively). *S. typhimurium* was only detected in the environment in flies and soil (20.0% each). While, *S. Dublin* was just detected in flies (20.0%).
10. Serotyping of *Salmonella* spp. in farm (II) proved that *Salmonella* isolation and identification was limited to environmental samples Furthermore *S. kentuky* was the most predominantly detected serotype (50.0%), followed by *S. enteritidis* and *S. typhimurium* (33.3 and 16.7%, respectively). Moreover *S. enteritidis* and *S. typhimurium* were detected only in flies' samples (36.4 and 18.2 %, respectively). was detected in flies. *S. kentuky* was detected in the highest percentage in swabs of attendants' hands (100.0%) followed by flies (45.5 %).
11. Regarding the risk factors associated with calf diarrhea in both farm (I and II) there were a variation between the two farms where in farm (I) there was a calving pen but not routinely cleaned and disinfected, the

management of newly born calves including umbilical care did not take place where some calves were suffering from hernia that affect their health condition and their ability to resist diseases. Calf housing was outdoor exposing them to different weather conditions, and in yard with earthy floor that was difficult to clean and disinfect with no drainage system leading to accumulation of manure under the animals contaminating their environment and aggravating of flies problem that act as a vector for disease pathogens. Mixing buffalo calves with cow calves consider a risk factor. Meanwhile, in farm (II) calves were kept with their dams for 2 weeks of life where they were naturally suckling their dams exposing them to different pathogens that contaminate their teat apices. Calves of different ages were housed together that increased the risk of diarrhea. Watering and feeding the animals from common water troughs, buckets and manager increased the probability of contamination of food and water and transmission of infection to healthy calves in both farms.

Results in part (I) revealed that miss managmental practice during raising of calves such over stocking density of calves, raising different ages together with different species, and raising of calves on earthy floor all that increased the risk of calves to the infection with enteropathogenic bacteria causing diarrhea, together with lower standard of hygiene and absence of routine disinfection program all that have led to higher frequency of enteropathogenic bacteria isolation from both calves and their environment particularly in farm (I).

Part II: Antimicrobial sensitivity pattern of enteropathogenic bacteria recovered from calves and their environment *in-vitro*

The antimicrobial sensitivity of identified enteropathogenic bacteria isolated from calves and their environment were tested *in-vitro* against 12 antibiotics and 4 different types of disinfectants commonly used in veterinary practice. Bacterial isolates showed resistance to 3 or more antimicrobial agents were selected for detection of antimicrobial resistance genes.

The obtained results showed that:

1. Antibiotic sensitivity testing of enteropathogenic bacteria recovered from calves and their environment in farm (I) showed that *Salmonella* spp. were significantly sensitive to enrofloxacin (63.2%), followed by florofenicol (52.6%), and erythromycin (52.6%). While they were significantly intermediately resistance to florofenicol (47.4%), neomycin (42.1%) and enrofloxacin (26.3%), and highly resistant (100.0% each) at $P<0.001$ to ampicillin, amoxicillin, penicillin, tetracycline, oxytertracycline, chloramphenicol, Sulfamethoxazole/trimethoprim complex and cefoxitin. *Shigella* spp. as well showed similar pattern to *Salmonella* spp. where they were significantly sensitive to enrofloxacin (68.7%), followed by florofenicol (25.0%) and significantly intermediately resistant to florofenicol (62.5%), followed by neomycin and erythromycin (50.0% each) then enrofloxacin (25.0%), while they were highly resistant (100.0% each) at $P<0.001$ to (β-lactamases), tetracycline, oxytertracycline, chloramphenicol, Sulfamethoxazole/trimethoprim complex and cefoxitin. Referring to *Klebsiella* spp. were significantly sensitive to enrofloxacin and florofencicol and neomycin (100.0, 100.0

and 40.0%, respectively), and significantly intermediately resistant to neomycin (60.0%), but they were significantly and completely resistant to other antibiotics (100.0%) at (P<0.001). Meanwhile, *C. perfringens* was significantly sensitive to ampicillin and tetracycline (42.8% and 14.3%, respectively), and intermediately resistant to tetracycline (21.4%), moreover it exhibited significant resistance to the rest of the used antibiotics (100.0%) at (P<0.001).

2. The results of antibiotic sensitivity testing in farm (II) showed that *Salmonella* spp. were only intermediately resistance to neomycin and enrofloxacin (50% each), and completely resistant the rest of the used antibiotics. While *Shigella* spp. exhibited significant sensitivity to neomycin followed by florofenicol (21.4 and 14.3%, respectively) and significant intermediate resistance to florofenicol and enrofloxacin (50.0, 42.9%, respectively) at (P<0.001) and significantly completely resistance (100.0% each) to ampicillin, amoxicillin, penicillin, tetracycline, oxytetracycline, chloramphenicol, cefoxitin, Sulfamethoxazole/trimethoprim and erythromycin. Meanwhile, *Klebsiella* spp. showed significant sensitivity just to neomycin (40.0%), and were significant intermediately resistance to neomycin (10.0%) at (P<0.001) and exhibited significantly complete resistance to all of the other antibiotics.
3. Antibiotic sensitivity testing of *E. coli* recovered from calves and their environment revealed that *E. coli* obtained from calves in farm (I) showed significant sensitivity to enrofloxacin, neomycin, chloramphenicol and florofenicol (80.0, 60.0, 30.0 and 30.0%, respectively), and significant intermediate resistance to erythromycin (50.0%) while they were significantly resistant (100.0%) to (β-lactamases), tetracycline, oxytertracycline, Sulfamethoxazole/trimethoprim complex and cefoxitin at (P<0.001).

Meanwhile the environmental isolates of *E. coli* was significantly sensitive to enrofloxacin and chloramphenicol (20.0 and 10.0%, respectively), and significantly intermediately resistant to neomycin, florofenicol, erythromycin and enrofloxacin (50.0, 50.0, 30.0 and 20.0%, respectively) while they showed significant complete resistance to (β-lactamases), tetracycline, oxytertracycline, Sulfamethoxazole/trimethoprim complex, florofenicol and cefoxitin at (P<0.001). Whilst *E. coli* isolates recovered from calves in farm (II) exhibited significant sensitivity to enrofloxacin, florofenicol and neomycin (33.3, 16.7 and 11.1%, respectively), and significant intermediate resistance to enrofloxacin, neomycin, florofenicol, oxtetracycline and chloramphenicol (66.6, 61.1, 50.0, 16.7 and 11.1%, respectively) and they were significantly resistant (100.0%) to the rest of tested antibiotics at (P<0.001). Moreover, environmental isolates of *E. coli* were significantly moderately sensitive to enrofloxacin and florofenicol (50% each), and were significantly intermediate resistant to chloramphenicol, erythromycin, neomycin, enrofloxacin, florofenicol and Sulfamethoxazole/trimethoprim (70.0, 60.0, 50.0. 50.0, 50.0 and 20.0%, respectively), while they significantly resistant to the rest of used antibiotics (100.0%) at (P<0.001).

4. Disinfectant efficacy testing against different bacterial isolates in farm (I) showed that Virkon® S (1%) was significantly efficient against *Salmonella* spp. (78.9 and 68.4%, respectively) at (P<0.05; 0.001) at 30 and 15 min exposure time. While iodine (5%) exhibited the least bactericidal effect (15.7%) at (P<0.001) after 15 min of exposure. Regarding *Shigella* spp. were significantly sensitive to Virkon® S (1%) followed by TH^{4+} (0.5%) at exposure time 30 (75.0 and 68.8%, respectively) then Virkon® S (1%) after 15min of exposure (50.0%) at (P<0.05), while H_2O_2 (1%) exhibited the least bactericidal effect (25.0

and 18.8%, respectively) at 30 min and 15min. Meanwhile *Klebsiella* spp. were significantly sensitive (100.0%) to Virkon® S (1%) after 30 min of exposure time, followed by TH $^{4+}$ (0.5%) at exposure time 30 min (80.0%) then Virkon® S (1%) after15min of exposure (70.0%) at (P<0.001) and the least efficiency was exhibited by iodine (5%) (6.7%) at contact time 5 min.

5. In farm (II) disinfectant sensitivity pattern showed that *Salmonella* spp. were mostly sensitive (75.0 and 50.0%, respectively) to Virkon® S (1%) at contact time 30 and 15min, and they were less sensitive (25.0 %) to $H_2 O_2$ (1%) at contact time 15 and 10 min. Meanwhile, *Shigella* spp. were significantly sensitive (78.6, 71.4 and 64.3%, respectively) at (P<0.05) to Virkon® S (1%) and TH $^{4+}$ (0.5%) at exposure time 30 and 15 min of exposure time to Virkon® S (1%), and the least sensitivity (21.4% each) was exhibited to H_2 O_2 (1%) at the same exposure time. Meanwhile, *Klebsiella* spp. were highly sensitive (100.0%) to Virkon® S (1%) after 30 min of exposure while after 15 min (76.6%) and (70.0%) sensitive to TH $^{4+}$ (0.5%) after exposure time 30min at (P<0.001; P<0.05, respectively) while H_2 O_2 (1%) had the lowest bactericidal effect (26.6 and 16.6%, respectively) at (P<0.001; P<0.05) at contact time 15 and 10 min.
6. Disinfectant sensitivity against *E. coli* isolates recovered from calves and their environment in farm (I and II) revealed *E. coli* isolates obtained from the calves in farm (I) were significantly sensitive (80.0 % each) at (P<0.001) to both TH^{4+} 0.5% and Virkon® S 1% after 30 min of exposure. On the other hand, environmental strains in farm (I) were significantly sensitive to Virkon® S 1% and iodine (5%) (70.0 each) after contact time 30min followed by Virkon® S 1% after 15 min (50%) at (P<0.001), while both *E. coli* isolated from the calves and their environment were significantly resistant (100.0%) to H_2 O_2 1%

after contact time 30 min at (P<0.001). Moreover, in farm (II) Virkon® S 1% showed highly significant bactericidal effect against *E. coli* isolates from the calves (100.0 and 83.3%, respectively) at 30 and 15 min of exposure at P<0.001 and sensitive to TH^{4+} 0.5% and iodine 5% (83.3% each) after 30 min of exposure, while H_2O_2 1% was significantly the lowest effective disinfectant (22.2%) at (P<0.001) after 5 min of exposure. Whilst the environmental strains were significantly sensitive to Virkon ® S 1% after 30 min and 15 min of exposure (80.0, 60.0%, respectively) and 70.0% iodine (5%) after 30 min of exposure at (P<0.001), and likewise they were significantly resistant (100.0%) to H_2O_2 1% after 30 min of exposure at P<0.001.

7. The distribution of antimicrobial resistance genes showed that bla_{SHV} and bla_{TEM} resistance gene; responsible for resistance to β-lactams antibiotics, were detected in 8 isolates of *E. coli* from the calves and their environment. Meanwhile, while bla_{OXA-1} resistance gene; responsible for resistance to β-lactams antibiotics, was detected in only one isolate of *E. coli* form the calves. On the other hand, d*f*rA resistance gene of trimethoprim was detected in 4 isolates of *E. coli* (2 of animal origin and 2 of environmental origin). The *qacED1*gene; responsible for resistance to quaternary ammonium compound (QAC) disinfectants, which was detected in 5 isolates of *E. coli* (3 of animal origin and 2 of environmental origin). Meanwhile, *floR* resistance gene that is responsible for resistance to phenicols was detected in only one isolate of *E. coli* isolated from diarrheic calves. The *sul1*resistance gene; responsible for resistance to sulfonamides and *tetA*(A) gene; responsible for resistance to tetracycline , were detected in 3 isolates of *E. coli* (2 were obtained from animals and 1 was obtained from environment).

The results in part (II) indicated that most of the isolated enteropathogenic bacteria from the calves or their environment exhibited high degree of resistance to the majority of the used antibiotics and this might be a consequence of indiscriminative use of antibiotics in clinical practice, also a variable degree of resistance to most of the disinfectants used although there were no routine disinfection and this might be due to improper cleaning and presence of organic matter that inactivate the disinfectants. All of the screened resistance genes were detected in the animal isolates pointing out to their possibility to act as a reservoir to such genes in the environment and risk to human population.