Prevalence and antimicrobial resistance of *Salmonella* isolates in Moroccan laying hens farms

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Primary Audience: Flock Supervisors, Quality Assurance Personnel, Researchers, Veterinarians

SUMMARY

Increasing emergence of salmonellosis presents a threat to the effective control of foodborne disease in humans. The purpose of this study was to evaluate the prevalence of drug susceptibility and molecular characteristics of non-typhoidal *Salmonella* (NTS) isolated from laying hens (LH) in 3 Moroccan regions, Rabat-Salé-Zemmour-Zaër (RSZZ), Souss-Massa-Drâa (SMD), and the grand Casablanca (GC). A total of 351 samples were collected from 30 consumer egg laying houses at the end of the egg laying period from April to July 2011. Sixty-four out of these 351 examined samples were contaminated by *Salmonella.* The *Salmonella* isolated strains were then serotyped and tested for drug susceptibility and analyzed by polymerase chain reaction (PCR) for the presence of the invasion-associated genes *invA* and *spvC* and nalidixic acid resistance-associated *qnr* gene. The prevalence of NTS infection in LH was estimated to be 73.3%. Seven *Salmonella enterica* serovars were identified: Enteritidis (37.5%), Kentucky (31.2%), Infantis (10.9%), Typhimurium (6.2%), Thompson (6.2%), Agona (4.6%), and Amsterdam (3.1%). Drug susceptibility testing showed that 65.6% of *Salmonella* were resistant to at least one antibiotic and 25% were resistant to ciprofloxacin. All isolates were positive for the invasion gene *invA* and 28% of them were positive for the virulence gene *spvC*. All nalidixic acid-resistant *S.* Enteritidis isolates were negative for *qnr* plasmid genes. Our findings clearly suggest the necessity to establish an NTS monitoring and control program for LH in Morocco.

Key words: *Salmonella*, laying hens, breeding, drug susceptibility, Morocco

2016 J. Appl. Poult. Res. 00:1–8
http://dx.doi.org/10.3382/japr/pfw036

DESCRIPTION OF PROBLEM

The ubiquitous *Salmonella* is a significant problem for public health and the poultry sector. *Salmonella* can establish a clinically unapparent infection of variable duration in laying hens (**LH**), which is significant as a potential zoonosis. Such animals may be important in relation to the spread of infection among flocks and as causes of human foodborne

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infection. Intensive epidemiology and laboratory investigations identified shell eggs as the major vehicle for *Salmonella* Enteritidis infection in humans, and they reported that eggs had been internally contaminated by transovarian transmission of *Salmonella* Enteritidis in the LH [1]. Food poisoning caused by *Salmonella* is often associated with consumption of eggs and/or other foods that use eggs as a component. Most of the time, infected LH are at the origin of these infections [2]. According to a survey conducted in the United States, each year 31 major pathogens cause 9.4 million episodes of foodborne illness, most of which are caused by norovirus (58%) followed by non-typhoidal *Salmonella* (**NTS**) (11%), although NTS was found to be the leading cause of hospitalization (35%) and death (28%) [3]. NTS was also found to be involved in approximately 65% of collective food poisoning cases in France [4]. In Morocco, *Salmonella, Staphylococcus aureus*, and *Clostridium perfringens* were reported to be responsible for, respectively, 42.8, 37, and 1.7% of food poisoning cases in humans [5]. Of the 1,577 cases of epidemic food poisoning reported annually in Morocco, *Salmonella* was confirmed in 96 cases, and suspected in 259 cases [6]. It is reported worldwide that all *Salmonella* serotypes (not only *S.* Typhi) show resistance to drugs [7]. In Morocco, resistance to quinolone and carbapenem was commonly detected in *S.* Kentucky, while *S.* Typhimurium showed a high level of resistance to the third generation of cephalosporin [8,9]. Currently, there are limited data on the prevalence of *Salmonella* in LH breeding in Morocco. The aim of this work was to evaluate the prevalence of NTS serotypes, antimicrobial susceptibility, and detection of *invA* and *spvC* genes in *Salmonella* isolates collected on LH farms in Morocco.

MATERIALS AND METHODS

Materials

Laying hen farms were randomly selected from a list of LH units authorized in Morocco gratefully provided by the National Office of Health Security of Food Products (ONSSA) (204 farms). Hens were aged between 14 to 83 wk old. Animals did not show any signs of disease (in particular diarrhea) and they were not subject to any antibiotic treatment during the phase of egg production. Samples were collected from LH groups that were at the end of the egg laying period (between 74 and 82 wk old). The geographical location and the importance of the LH farm density were taken into consideration. Four big regions were identified in Morocco (Table 1). However, due to time constraints, samples were collected only from farms in the 3 biggest and most populated regions in Morocco, Grand Casablanca **(GC),** Rabat-Salé-Zemmour-Zaër **(RSZZ)**, and the Souss-Massa-Drâa **(SMD)**. A total of 351 samples were collected from April to July 2011, from 30 visited LH farms (Table 2). Twelve LH farms were visited in RSZZ, a region located in the northwest coastal region of Morocco. This region includes the capital city and its outskirts (over 9,580 km^2, with a population of about 2,676,754 inhabitants). A total of 150 samples were collected from these LH farms [60 fresh dropping samples, 24 dust samples (*i.e.*, samples collected from

Table 1. Distribution of provinces, number of farms authorized in Morocco, their production capacity, and the number of visited farms by region.[1]

Region	Provinces included in each region	Number of farms	Production capacity (number of laying hens)	Number of visited farms
Grand Casablanca	Casablanca, Nouaceur, Mediouna, Mohammedia, El Jadida and Settat	94	8,416,400	10
Rabat-Salé-Zemmour-Zaër	Khémisset, Salé and Skhirat-Temara	76	7,533,600	12
Souss-Massa-Drâa	Chtouka, Taroudant and Inezgane	14	851,000	8

[1]Information about laying hen units authorized in Morocco was provided by the National Office of Health Security of Food Products (ONSSA) in 2011.

Table 2. Resistance profile, serotype, plasmid, *invA*, and *spvC* gene of 64 *Salmonella* isolates from laying hen farms in Morocco.

Region	Farm number	Date	Sample	Resistance profile	Serotype	Plasmid sizes (kb)	*invA* PCR	*spvC* PCR
	1	15/04/2011	Droppings	Susceptible	Amsterdam	–	+	–
		15/04/2011	Droppings	Nal	Enteritidis	54–2.7	+	+
		15/04/2011	Droppings	Nal	Enteritidis	54–2.7	+	+
		15/04/2011	Food	Nal	Enteritidis	54–2.7	+	+
	4	15/04/2011	Food	Nal	Enteritidis	54–2.7	+	+
Rabat-Salé-Zemmour-Zaër	5	19/04/2011	Droppings	Susceptible	Infantis	–	+	–
		19/04/2011	Droppings	Susceptible	Infantis	–	+	–
n = 150		19/04/2011	Droppings	A;Cf;S;Sul;Nal;Cip;Gm	Kentucky	2.1	+	–
	6	19/04/2011	Droppings	A;Cf;S;Sul;Nal;Cip;Gm	Kentucky	2.7	+	–
		19/04/2011	Dust samples[1]	Susceptible	Infantis	–	+	–
		19/04/2011	Food	Susceptible	Infantis	–	+	–
		19/04/2011	Food	Susceptible	Infantis	–	+	–
		22/04/2011	Droppings	Te;Nal;Cip;Sul;Gm	Kentucky	–	+	–
	8	22/04/2011	Food	Te;Nal;Cip;Sul;Gm	Kentucky	–	+	–
		22/04/2011	Food	Te;Nal;Cip;Sul;Gm	Kentucky	–	+	–
	9	29/04/2011	Droppings	Susceptible	Amsterdam	–	+	–
Souss-Massa-Daraa		Mai 2011	Droppings	Nal	Enteritidis	54	+	+
	13	Mai 2011	Droppings	Nal	Enteritidis	54	+	+
n = 88		Mai 2011	Droppings	Susceptible	Enteritidis	54–5.6	+	+
	14	Mai 2011	Dust samples[1]	Nal	Enteritidis	54	+	+
		Mai 2011	Dust samples[1]	Nal	Enteritidis	54	+	–
	15	Mai 2011	Droppings	Nal	Enteritidis	54–5.6	+	+
		Mai 2011	Droppings	Nal	Enteritidis	54–5.6	+	+
		Mai 2011	Dust samples[1]	Susceptible	Thompson	2.7	+	–
		Mai 2011	Dust samples[1]	Susceptible	Thompson	2.7	+	–
		Mai 2011	Cloacal swabs	Susceptible	Thompson	2.7	+	–
		Mai 2011	Cloacal swabs	Susceptible	Thompson	2.7	+	–
	17	Mai 2011	Droppings	A;Cf;Te;Nal;Cip	Kentucky	2.1	+	–
		Mai 2011	Droppings	A;Cf;Te;Nal;Cip	Kentucky	2.1	+	–
		Mai 2011	Droppings	A;Cf;Te;Nal;Cip;S	Kentucky	2.1	+	–
	18	Mai 2011	Droppings	A;Cf;Te;Nal;Cip;S	Kentucky	2.1	+	–
		Mai 2011	Dust samples[1]	A;Cf;Te;Nal;Cip;S	Kentucky	2.1	+	–
		Mai 2011	Dust samples[1]	A;Cf;Gm;S;Te;Nal;Cip;Sul	Kentucky	2.1	+	–
	19	Mai 2011	Droppings	Nal	Enteritidis	54	+	+
		Mai 2011	Droppings	Nal	Enteritidis	54	+	+
		Mai 2011	Dust samples[1]	Nal	Enteritidis	54	+	+
	20	Mai 2011	Droppings	A;Sul;Te	Typhimurium	–	+	–
		Mai 2011	Droppings	A;Sul;Te	Typhimurium	–	+	–
		Mai 2011	Cloacal swabs	A;S;Te; Sul	Typhimurium	5.6	+	–
Grand Casablanca		29/06/2011	Droppings	Susceptible	Infantis	–	+	–
	21	29/06/2011	Food	A;Cf;S;Te;Nal;Cip;Sul	Kentucky	5.6	+	–
n = 113		29/06/2011	Water	Susceptible	Kentucky	5.6	+	–
		29/06/2011	Dust samples[1]	Susceptible	Kentucky	5.6	+	–
	22	29/06/2011	Droppings	Susceptible	Kentucky	5.6	+	–
		29/06/2011	Droppings	Susceptible	Agona	–	+	–
		29/06/2011	Dust samples[1]	Susceptible	Agona	–	+	–
		29/06/2011	Food	Susceptible	Agona	–	+	–

Table 2. *(Continued.)*

Region	Farm number	Date	Sample	Resistance profile	Serotype	Plasmid sizes (kb)	*invA* PCR	*spvC* PCR
	23	29/06/2011	Droppings	A;Cf;S;Te;Nal;Cip;Sul	Kentucky	5.6–2.7	+	–
		29/06/2011	Droppings	A;Cf;S;Te;Nal;Cip;Sul	Kentucky	2.7	+	–
		29/06/2011	Droppings	A;Cf;S;Te;Nal;Cip;Sul	Kentucky	2.7	+	–
	24	29/06/2011	Dust samples[1]	Nal	Enteritidis	54	+	+
		29/06/2011	Droppings	Nal	Enteritidis	54	+	–
	25	29/06/2011	Droppings	Nal	Enteritidis	54–5.6	+	+
	26	29/06/2011	Droppings	Nal	Enteritidis	54–5.6	+	–
		29/06/2011	Droppings	Nal	Enteritidis	54–5.6	+	+
	27	22/07/2011	Dust samples[1]	A;Cf;S;Te;Nal;Cip;Sul	Kentucky	54	+	–
	28	22/07/2011	Droppings	Nal	Enteritidis	54	+	+
		22/07/2011	Droppings	Nal	Enteritidis	54	+	+
		22/07/2011	Droppings	Nal	Enteritidis	54	+	–
		22/07/2011	Droppings	Nal	Enteritidis	54	+	–
	29	22/07/2011	Droppings	Nal	Enteritidis	54	+	–
		22/07/2011	Droppings	Susceptible	Typhimurium	54	+	–
		22/07/2011	Water	Susceptible	Infantis	–	+	–
	30	22/07/2011	Droppings	Susceptible	Kentucky	–	+	–

A: amoxicillin; Cf: cephalothin; Te: tetracycline; Amc: amoxicillin + clavulanic acid; S: streptomycin; Sul: sulfonamides; SXT: sulfonamides + trimethoprim; Gm: gentamicin; Nal: nalidixic acid; Cip: ciprofloxacin;- : Not found; + : presence.
[1]Dust samples: Sampling is done by collecting dust from exhaust fans, screens, and other equipment in the poultry house.

exhaust fans, screens, and other equipment in the poultry house), 12 pooled cloacal swab samples, 12 food samples, 12 water samples, and 30 dead animal organs (liver, spleen, oviduct, and ceca)] from April to July 2011. Ten LH farms were selected in the GC, a region considered as being the most densely populated region in the country. It is located in the northwest of Morocco and covers 1,615 km^2 with 4,270,750 inhabitants. A total of 88 samples were collected from these LH farms [40 fresh dropping samples, 16 dust samples, 8 pooled cloacal swab samples, 8 food samples, 8 water samples, and 8 dead animal organs (liver, spleen, oviduct, and ceca)] from April to July 2011. In the third studied region, SMD, located in the south of Morocco, 8 LH farms were visited. This region includes the Souss Valley, a part of the anti-atlas mountains and the region of Ouarzazate. It covers an area of 70,880 km^2 and has a population of 3,113,653 inhabitants. A total of 113 samples were collected from these LH farms [50 fresh dropping samples, 20 dust samples, 10 pooled cloacal swab samples, 10 food samples, 10 water samples, and 13 dead animal organs (liver, spleen, oviduct, and ceca)] from April to July 2011.

Isolation and Identification of Salmonella

The isolation and microbiological characterization of *Salmonella* were performed according to "Association Française de Normalisation" (AFNOR) (NF U 47-100) [10]. Suspected colonies were subjected to oxidase and urease tests, followed by bacterial identification using the API 20^E systems (Bio Mérieux R SA, Marcy-l'Etoile, France). The molecular confirmation of *Salmonella* strains was performed by amplification of the 275-bp fragment of the *invA* gene (Accession number M90846.1) using the primer pair: Forward (5′-tatcgccacgttcgggcaa-3′) and reverse (5′-tcgcaccgtcaaaggaacc-3′) [11]. The amplification program consisted of an initial denaturation at 95°C for one min followed by 30 cycles of 95°C for 45 sec, 58°C for 30 sec, and 72°C for 45 sec and a final extension at 72°C for 10 minutes. The PCR product was then analyzed by electrophoresis on agarose gel and visualized under ultraviolet transillumination after ethidium bromide staining. An extracted DNA from *Salmonella* Typhimurium ATCC14028 reference strain was used as positive control.

Salmonella Strains Serotyping

Salmonella isolates serotyping was performed using the slide agglutination test with specific antisera raised against "0" and "H" antigens of *Salmonella* (BioRad, Marnes-La-Coquette, France). *Salmonella* enterica serotype was determined according to the White-Kauffmann-Le Minor classification scheme [12].

Drug Susceptibility Testing

Antibiotic susceptibility testing was performed using the disc diffusion method on Mueller-Hinton agar (**MHA**) and results were interpreted according to the EUCAST breakpoints (Committee of the French Society for Microbiology (CA-SFM)). The strains were screened for their resistance to the following antibiotics (Bio-Rad): Amoxicillin, Amx 25 μg; cefalotin, Cf 30 μg; amoxicillin-clavulanic acid, Amc 20 + 10 μg; cefoxitin, Fox 30 μg; cefotaxime, Ctx 30 μg; ceftriaxone, Cro 5 μg; ceftazidime, Caz 30 μg; chloramphenicol, C 30 μg; streptomycin, S 10 μg; gentamycin, Gm 30 μg; trimethoprime, Tmp 5 μg; sulfonamids, Sul 200 μg; nalidixic acid, Na 30 μg and ciprofloxacin, Cip 5 μg. *E. coli* ATCC 25922 was used as a quality control strain.

Plasmid Extraction

Plasmid DNA extraction was performed by a rapid alkaline lysis procedure [13]. The plasmid DNA was analyzed by electrophoresis on a 0.75% (w/v) agarose gel after running in 0.5× TBE buffer [10 mM Tris, 0.4 mM Boric Acid and 1.0 mM EDTA (pH8)] at 50 mA (120 volts) for 2 h at room temperature and then visualized by UV illumination. Plasmid extract from *Escherichia* coli V517 strain carrying 8 plasmids (54 kb, 7.2 kb, 5.6 kb, 5.1 kb, 3.9 kb, 3.0 kb, 2.7 kb and 2.1 kb) was used as a molecular weight marker.

SpvC Genes Detection

The DNA of *Salmonella* isolates was prepared by the boiling method. Approximately a loopful of culture was taken from tryptic soy agar cultures (18 to 24 h at 37°C) and placed in sterile microcentrifuge tubes containing 100 μL of sterilized DNAse-free and RNAse-free milliQ water (Millipore, Bedford, MA), vortexed, and samples were heated at 100°C for 10 minutes. Cell debris was removed by centrifugation at 12,000 × g for 15 min and 2.5 μL of the supernatant was used as a DNA template in polymerase chain reaction (PCR) mixture. PCR was performed with one set of specific primer pairs: Forward (5′-cggaaataccatcaaata-3′) and reverse (5′-cccaaacccatacttactctg-3′), for the invasion gene *spvC*; this primer pair was predicted to yield a 669-bp product [11]. PCR products were resolved by electrophoresis in 1.5% agarose gel and visualized under ultraviolet transillumination after ethidium bromide staining. *Salmonella* Typhimurium ATCC14028 and *E. coli* HB101 were used as positive and negative controls, respectively.

Plasmid qnr Genes (A, B, and S) Detection

All *Salmonella* Enteritidis nalidixic acid-resistant isolates were analyzed by multiplex-PCR to detect the presence of plasmid *qnr* genes (A, B, and S) associated with quinolone resistance as described previously [14].

RESULTS AND DISCUSSION

Prevalence of Salmonella

A farm is considered infected if *Salmonella* is isolated from at least one collected sample. In this study, 76.7% (23/30) of the visited farms were contaminated by *Salmonella.* The high contamination rate by *Salmonella* is comparable to the prevalence found in some European countries, such as Spain (73.2%), Portugal (79.5%), and Poland (77.2%) [15]. The contamination rate of the visited farms was: 100% (10/10) in GC, 87.5% (7/8) in SMD, and 50% (6/12) in RSZZ. The PCR of the *invA* gene has been recognized as an international standard for the detection of the genus. Therefore, this method is a powerful tool for an efficient *Salmonella* diagnosis [16]. Of the total of 351 samples collected from the 30 visited LH farms, 18.2% (64/351) were

contaminated by *Salmonella* (Table 2). Our results further showed the presence of the *invA* gene in all *Salmonella* isolated from LH in Morocco. This finding was consistent with previous reports [17] that established the presence of *invA* gene in nearly all *Salmonella* strains irrespective of serovar or source.

Our study further showed that the highest contamination rate by *Salmonella* was found in dropping samples (61%), followed by dust samples (18%), food samples (12.5%), cloacal swabs (4.6%), and water samples (3.1%) (Table 2). All dead animal organs analyzed for *Salmonella* were, however, found negative. This high frequency of *Salmonella* may be due to applied biosecurity measures (no showering and/or changing facilities for visitors and staff entering poultry houses were available) and the absence of quality control of drinking water in the visited farms.

Serotype of Salmonella

Among 64 *Salmonella* isolates and according to the Kauffmann-White scheme described previously, 7 different serotypes were identified. The serotype distribution was as follows: Enteritidis 37.5% (24/64), Kentucky 31.3% (20/64), Infantis 10.9% (7/64), Typhimurium 6.2% (4/64), Thompson 6.2% (4/64), Agona 4.7% (3/64), and Amsterdam 3.1 % (2/64) (Table 2). Our results showed a predominance of Enteritidis serotype, although this remains lower than that found in LH breeding in the European Community (57.5%) in 2002 [15].

Resistance to the Antimicrobial Agents

As shown in (Table 2), antibiotic resistance of the isolated *Salmonella* strains to 14 antimicrobial agents showed a high percentage of resistance to the following antimicrobial agents: Nalidixic acid (61%), ciprofloxacin (25%), amoxicillin (21%), tetracyc1in (25%), cefalotin (25%), streptomycin (18%), sulfonamides (14%), and gentamycin (8%). Forty-two strains out of 64 (65.6%) were resistant to at least one tested antimicrobial agent. Furthermore, a high prevalence of multiresistance among *Salmonella* strains was observed. In fact, 19 out of 64 *Salmonella* strains were resistant to 2 or more antimicrobial agents. *Salmonella* Kentucky showed the highest level of resistance (25%) to the different tested drugs, followed by *Salmonella* Typhimurium (4.6%). These data were comparable to previous data [11]. *Salmonella* Enteritidis showed 37.5% of resistance only to nalidixic acid. This result was in accordance with previous data [18]. The remaining serotypes, Amsterdam, Agona, and Infantis, were sensitive to all tested antibiotics. It is, however, noteworthy that all visited regions were contaminated by *S*. Kentucky strains resistant to ciprofloxacin. This result is in agreement with those of a recent epidemiological investigation conducted in France [19]. National health, food, and agricultural authorities should include *Salmonella* Kentucky resistant to ciprofloxacin among the strains targeted in national programs to control *Salmonella* in poultry.

Plasmids

The *Salmonella* isolated showed different plasmid profiles with plasmid sizes ranging from 2.1 to 54 kb. Seventeen isolates of *Salmonella* did not show any plasmids, while 47 strains (73.5%) were carrying plasmids (2.1 to 54 kb). All nalidixic acid-resistant strains of *Salmonella* Enteritidis were found to carry one plasmid (54 kb) (Table 2). Several investigators reported that resistance to different antimicrobial agents was mediated by a large plasmid [11]. This plasmid whose size is greater than or equal to 90 kb was not found in our study.

Occurrence of spvC Gene

The detection of *spvC* invasion-associated gene by PCR showed that 78% (18/23) of S. Enteritidis resistant to nalidixic acid were found positive for *spvC*, while the other serotypes in our collection were negative (Table 2). The *spvC* gene is probably located on the plasmid (∼54 kb) (Table 2). Similar data were reported for minced turkey meat in Casablanca [11]. Abouzeed and collaborators showed that the *spvC* gene was detected in 7 human isolates but was not detected in chicken or bovine isolates [17].

Plasmid qnr Genes (A, B, and S) Detection

As the *qnr* gene is known to confer a low level of resistance to both quinolones and fluoroquinolones, all of the 23 S. Enteritidis resistant to nalidixic acid were subjected to multiplex *qnr* PCR amplification and were found negative. This finding may be due to other mechanisms such as chromosomal mutations of quinolone resistance determining region (**QRDR**) of the DNA gyrase and topoisomerase IV. It is, however, interesting to note that a recent study reported the presence of the plasmid *qnrS* gene in *Enterobacter* cloacae and *Klebsiella* pneumoneae isolated from humans at Ibn-Rochd Hospital-Casablanca [14].

CONCLUSION AND APPLICATIONS

1. In this study, the prevalence of NTS infection on LH farms was estimated to be 73.3%.
2. Seven *Salmonella enterica* serovars were identified: Enteritidis (37.5%), Kentucky (31.2%), Infantis (10.9%), Typhimurium (6.2%), Thompson (6.2%), Agona (4.6%), and Amsterdam (3.1%).
3. In this study, 65.6% of *Salmonella* strains were resistant to at least one antibiotic, and 28% (18/64) of them were positive for the *spvC* invasion-associated gene, probably carried by a plasmid (54 Kb).
4. This study showed an impressive implant of the *S.* Kentucky resistant to the ciprofloxacin on all visited farms.
5. In light of these findings, we recommend the establishment of a strategy to improve the current situation by the implementation of a surveillance and control program of *Salmonella* on LH farms and extending the collected samples (from breeding to fresh droppings and samples of dust). This will provide more data for conducting risk analysis with relevance to human and animal health.

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Acknowledgments

This work was supported by Institut Pasteur du Maroc (IPM), "Division of Pharmacy and Veterinary Inputs, Control and Expertises Service (DPIV); and Agronomic and Veterinary Institute Hassan II Rabat, Morocco." All authors would like to thank Doctor Khadija ID Sidi Yahia, head of (DPIV), Professor Naima ELmdaghri, director of IPM, Khyatti Meriem, PhD, head of research department of IPM, and Akil abdellah, PhD, for their support and assistance.